The effects of prostaglandin $F_{2\alpha}$ on the force / calcium relationship in pregnant rat myometrium

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By

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Abstract

Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) is a myometrial stimulant, both $PGF_{2\alpha}$ and its receptor are reported to increase towards parturition. With high $PGF_{2\alpha}$ levels correlating with pre-term birth, especially those caused by infection. The mechanism by which $PGF_{2\alpha}$ exerts its affects on uterine excitation-contraction coupling is unknown.

The aim of this work was to describe the relationship between force and $[Ca^{2+}]_i$ in pregnant rat myometrium and the effects of $PGF_{2\alpha}$ on this relationship. The mechanism by which $PGF_{2\alpha}$ exerts its affects was also investigated, focused on the role of Ca^{2+} entry mechanisms. $PGF_{2\alpha}$ was examined on both longitudinal strips and isolated myocytes from pregnant Wistar rats. Strips were loaded with the Ca^{2+} sensitive indicator Indo-1AM and simultaneous recording of $[Ca^{2+}]_i$ and force were made using a photometric system combined with force measurements, at a sampling rate of 1KHz to get good temporal resolution. Cells were isolated using Liberase Blendzyme 3, loaded with Fluo-4AM, and $[Ca^{2+}]_i$ recorded using a Nipkow Disk based confocal imaging system.

Pregnant myometrial tissue gave three contractility patterns in control conditions; two spontaneously active, giving either irregular contractions, or smooth phasic contractions, while the third was not spontaneously active but responsive to high-K^+ stimulation. All contractions were preceded by a rise in $[Ca^{2+}]_i$ which was dependent upon Ca^{2+} entry through VOCC, while synchronisation was dependent upon gap junctions.

$PGF_{2\alpha}$ increased myometrial contractility. In spontaneous tissue there were two responses; firstly an increase in amplitude, duration and frequency of phasic contraction while the second resulted in a tonic-like contraction which lasted for the duration of agonist

application. On quiescent tissue, $PGF_{2\alpha}$ resulted in the imitation of spontaneous activity, which ceased upon removal of the agonist. $[Ca^{2+}]_i$ mirrored force in respect to frequency and duration, but $PGF_{2\alpha}$ did not increase the amplitude of Ca^{2+} transient above that seen under normal spontaneous activity. The increase in amplitude of force induced by $PGF_{2\alpha}$ is caused by an increase in the frequency of Ca^{2+} spikes within the Ca^{2+} spike burst.

$PGF_{2\alpha}$ resulted in the oscillatory release of Ca^{2+} from the SR in the form of propagating Ca^{2+} waves, initiated at one end of the cell. Re-admission of external Ca^{2+} resulted in activation of a nifedipine-resistance Ca^{2+} influx sensitive to La^{3+}, which suggests that the stimulant action of $PGF_{2\alpha}$ is associated with activation of a Ca^{2+}-release Ca^{2+}-entry coupling mechanism leading to opening of a SOCE pathway. In addition to this $PGF_{2\alpha}$ increase both force and $[Ca^{2+}]_i$ when applied in the presence of CPA and nifedipine, suggesting that $PGF_{2\alpha}$ also works in part through a receptor operated and / or non-selective cation channels.

Abbreviations

[ ]	Concentration
0Ca	Zero calcium
$[Ca^{2+}]_i$	Cytosolic calcium concentration
18-β-GA	18-β-glycyrrhetinic acid
ADP	Adenosine diphosphate
AM	acetoxymethyl
ATP	Adenosine triphosphate
BK_{Ca}	Large conductance potassium channel
˚C	Degrees celcius
Ca^{2+}	Calcium
CCE	Capacitative calcium entry
CCh	Carbachol
Cl^-	Chloride
Cl_{Ca}	Calcium activated chloride channel
CPA	Cyclopiazonic acid
DAG	Diacylglycerol
DMSO	Dimethyl sulfoxide
EGTA	Ethylene glycol tetraacetic acid
ER	Endoplasmic reticulum
FP	Prostaglandin $F_{2\alpha}$ receptor
Gd^{3+}	Gadolinium
GPCR	G-protein coupled receptor
HBSS	Hank's balanced salt solution
HEPES	*4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid*)

High-K^+	High potassium saline solution
I_{CRAC}	Calcium release activated calcium current
I_K	intermediate potassium channels
IP_3	inositol 1,4,5-trisphosphate
IP_3R	inositol 1,4,5-trisphosphate receptor
I_{SOC}	Store operated current
I.Q.R	Interquartile range
K^+	Potassium
KATP	ATP-sensitive potassium channel
KB	Kraft-Brühe
K_{Ca}	Calcium activated potassium channel
La^{3+}	Lanthanum
Mg^{2+}	Magnesium
MLC_{20}	Myosin light chain 20
MLC_{20}-P	Phosphorylated myosin light chain 20
MLCK	Myosin light chain kinase
MLCP	Myosin light chain phosphatase
N.A.	Numerical aperture
Na^+	Sodium
NSCC	Non-selective cation channel
NSCE	Non-selective cation entry
PG	Prostaglandin
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PGDH	Prostaglandin dehdrogenase
PDHS	prostaglandin endoperoxide H synthase
PIP_2	Phosphatidylinositol 4,5-bisphosphate

PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PSS	Physiological saline solution
RNA_i	Small interfering RNA
ROC	Receptor operated calcium channel
ROCE	Receptor operated calcium entry
RyR	Ryanodine receptor
S.E.M.	Standard error of the mean
SERCA	The sarco / endoplasmic reticulum Ca^{2+}-ATPase
S_K	Small potassium channel
SOCC	Store operated calcium channel
SOCE	Store operated calcium entry
TRP	Transient receptor potential
SR	Sarcoplasmic reticulum
VOCC	Voltage operated calcium channels

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Chapter 1

General Introduction

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General introduction

1.1 The Uterus

The uterus is the pivotal organ responsible for reproduction, it is responsible for the implantation of the fertilised embryo, where it then sustains the growing foetus and at term the myometrium becomes active in order to expel the foetus. The human uterus is a pear shaped organ, around 6-8cm long in the non-pregnant state, and consists of three layers; the serosa, endometrium, and the smooth muscle layer the myometrium. Despite the big difference between the human and Wistar rat (*Rattus norvegicus*) in gross anatomy, at the myometrial level they are remarkably similar. The human uterus is a simplex, consisting of one single organ, while the uterus of the Wistar rat along with other rodents and primitive mammals is duplex. As such they possess two uterine horns, each leading from an ovary to the vagina, allowing for multiple offspring (Figure 1.1.a).

1.1.1 The myometrium

The myometrium is the smooth muscle potion of the uterus is composed of both longitudinal and circular smooth muscle layers. The longitudinal layer consists of bundles of smooth muscle cells that are orientated parallel to the long axis of the uterus, while the circular layer is arranged concentrically to the longitudinal axis, next to the endometrium (Figure 1.1b) They have very different physiological and pharmacological characteristic, in regards to their electrophysiological and mechanical properties, especially in rat. These

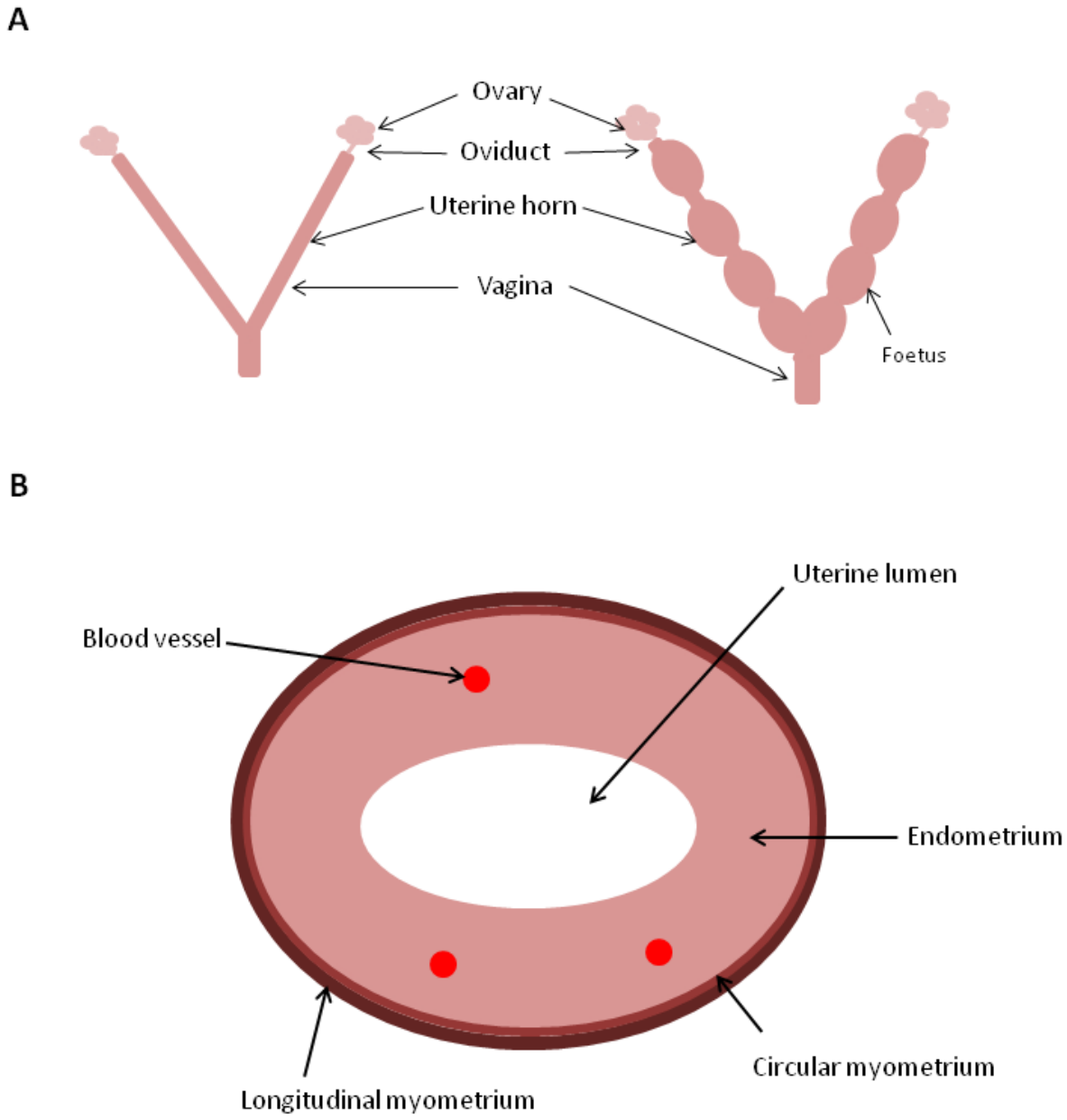

Figure 1.1 Schematic diagram of the rat uterus. (A) Gross anatomy of the rat uterus, non-pregnant and pregnant. (B) Cross-sectional area of non-pregnant rat uterus, indicating the two muscle layers; longitudinal and circular smooth muscle.

differences are apparent during gestation, towards parturition characteristics of the two layers become increasingly similar.

Smooth muscle cells of the myometrium are long, and spindle shaped, whose size is dependent upon pregnancy state and the stage of gestation. It is generally agreed that they are around 5-10 μm in diameter and 300-600 μm in length, although this is dependent on the state of the cell; contracted cells are shorter, but thicker in the middle, whereas relaxed and distended cells are long and narrow. To be able to function and produce adequate force, myocytes are organised into bundles. The cells within a bundle are connected via gap junctions to one another in series and in parallel, allowing the muscle bundle to act as a single unit, and so produced more force.

In order for the uterus to function, the uterus needs to grow to accommodate the growing foetus. The myometrium does this by both hyperplasia, an increase in number and hypertrophy, an increase in the size of individual myocytes. At the beginning of gestation this is achieved primarily by hyperplasia, hypertrophy taking over towards the latter part of the first half of gestation, while growth in the second half of pregnancy is the result of stretch.

At term, the myometrium needs to switch from a relatively inactive quiescent state, to one that is able to produce appropriate contractions. Too powerful contractions will cause foetal hypoxia and distress, while labour will be dysfunctional if contractions are too weak or uncoordinated. The myometrium achieves this by modulation of membrane potential and the propagation of action potential and Ca^{2+} signals throughout gestation.

1.2 Myometrial ion channels and transporters

For schematic overview of myometrial ion channels and transporter see Figure 1.2.

1.2.1 Ca^{2+} influx mechanisms

1.2.1.1 Voltage operated Ca^{2+} channels

Voltage operated Ca^{2+} channels (VOCC) are the principal mechanism of Ca^{2+} influx in spontaneously active myometrial tissue. The family of VOCC is comprised of six subtypes; L, N, P, Q, R and T types , of which L-type VOCC play a vital role in the generation of phasic contractility of the uterus, with the L-type VOCC blocker, nifedipine causing the abolishment of action potential, Ca^{2+} transient and the generation of force (Kuriyama et al. 1995; Shmigol et al. 1998b). T-type VOCC have also been suggested to be important to the uterus, although evidence of their physiological role in pregnant rat myometrium are contradictory (Amedee et al. 1987; Sperelakis et al. 1992; Young et al. 1993).

L-type VOCC have a Ca^{2+} specific voltage sensitive inward current, with a threshold activation of around -40mV and are fully activated when the membrane is depolarised to more than -10mV (Jmari et al. 1986) and exhibit a slow voltage- dependent inactivation (Honore et al. 1989; Sperelakis et al. 1992). While L-type VOCC are controlled by voltage, they are also rapidly desensitised to sustained depolarisation, explaining the classic response to high-K^+ depolarisation. Upon high-K^+ stimulation there is an increase in $[Ca^{2+}]_i$, which rapidly falls to a lower maintained level, it is the smaller noninactivating current that is responsible for the maintained $[Ca^{2+}]_i$ during sustained depolarisation, rather than the full initial current (Fleischmann et al. 1994).

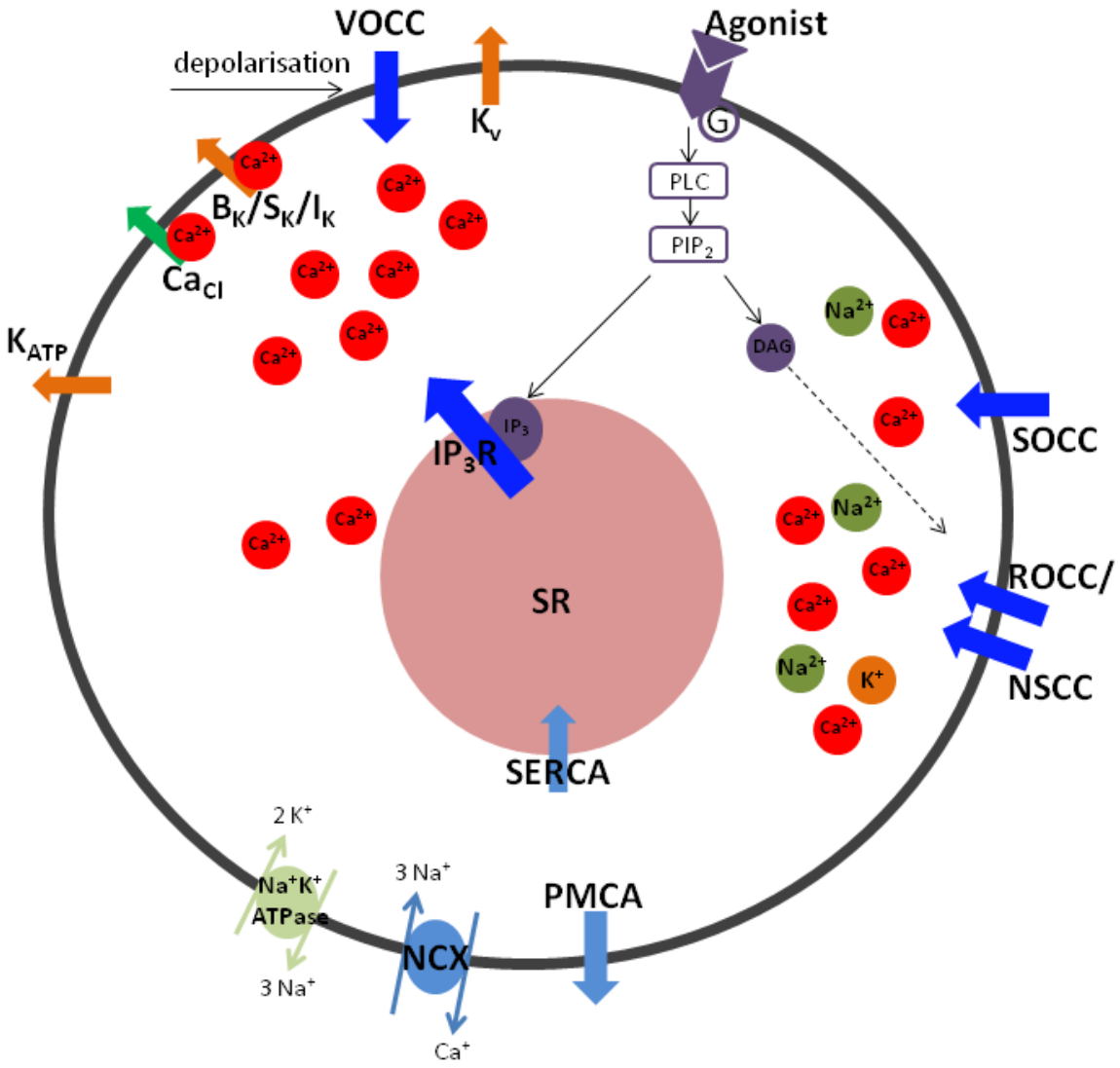

Figure 1.2 Schematic diagram depicting principal ion channels and transporters in rat myometrium. Arrows denote movement of ions across membrane. Ca^{2+} influx mechanisms denoted in dark blue, efflux pathways in pale blue and K^+ channels in orange. Black arrows are known pathways and dashed arrows represent a currently unknown pathway.

As L-type VOCC are voltage sensitive, they are modulated by all factors that influence membrane potential, allowing agonists to influence $[Ca^{2+}]_i$ by altering the current through L-type VOCC, which is primarily achieved through activation of Ca^{2+}-activated K^+ (K_{Ca}) or Cl^- (Cl_{Ca}) channels (Arnaudeau et al. 1994; Zhou et al. 2007). In addition to being modulated by voltage, L-type VOCC are inactivated by increasing concentrations of Ca^{2+} and Mg^{2+} (Jmari et al. 1986; Sanborn 1995). Agonists may also modulate L-type VOCC in myometrial smooth muscle, with oxytocin a principal uterotonic suggested to work in part through modulation of L-type VOCC, although this remains controversial (for review see Sanborn et al. 1998).

1.2.1.2 Non-selective cation channels / receptor-operated Ca^{2+} channels

Unlike L-type VOCC, which have received lots of attention and whose mechanisms have generally been elucidated, there is still confusion regarding the existence, function and mechanism of non-selective cation channels (NSCC) and receptor-operated Ca^{2+} channels (ROC).

In uterine smooth muscle, a non-selective cation channel has been suggested, being permeable to Ca^{2+}, Na^+ and K^+ , and activated independently of agonist stimulation (Miyoshi et al. 2004). However, classification and properties of NSCC and ROC and currents generated by agonists or their downstream effectors in uterus is still controversial. In response to agonists binding to their receptors or activation by downstream effectors, both NSCC and ROC demonstrate poor Ca^{2+} selectivity and are often permeable to multiple ions (Benham 1989; Kuriyama et al. 1995). These characteristic of NSCC and ROC result in confusion

regarding the differentiation between two, and so in this thesis will be considered as one entity.

There are a number of lines of evidence to suggest that NSCC and ROC mediate a voltage-independent Ca^{2+} influx mechanism, and thus can be differentiated from VOCC (Murray et al. 1991). NSCC and ROC can also be distinguished from store operated Ca^{2+} channels (SOCC) as neither the depletion of the SR (Wang et al. 1991; Guibert et al. 1999) nor inhibition of IP_3 receptors by heparin prevent activation of NSCC or ROC by agonists (Albert et al. 2003c; Zholos et al. 2004). While NSCC and ROC constitute an independent Ca^{2+} influx pathway, due to their heterogeneous nature, there is currently no conformity regarding the mechanism of activation, current or channels involved in NSCC and ROC response in either spontaneous or agonist induced myometrial contractility.

1.2.1.3 Store operated Ca^{2+} channels (SOCC)

Store operated Ca^{2+} entry (SOCE), historically known as capacitative calcium entry (CCE), was identified over a quarter of a century ago, suggesting that a specific plasma membrane channel is activated upon agonist stimulated depletion of the SR, resulting in an influx of Ca^{2+} (Putney 2011). SOCE has been identified in many cell types; including both excitable and non-excitable cells.

There are multiple lines of evidence to suggest the existence of SOCE in different cells including the uterus, based primarily on measurement of $[Ca^{2+}]_i$ and force. Firstly by measuring $[Ca^{2+}]_i$, depletion of the store in the absence of external Ca^{2+} substantially increases basal $[Ca^{2+}]_i$ upon re-admission of Ca^{2+} to the extracellular media; this increase in

basal $[Ca^{2+}]_i$ is nifedipine resistant and can be induced by cyclopiazonic acid (CPA) and thapsigargin (SERCA pump inhibitors), excluding ROC / NSCC involvement. This evidence along with the use of non-selective inhibitors of SOCE, such as La^{3+} and SKF96365, have been taken to be highly suggestive of SOCE, and found in many smooth muscle types; colonic (Kovac et al. 2008), cultured A10 cells (Xuan et al. 1992), coronary arteries (Wagner-Mann et al. 1992), gallbladder myocytes (Morales et al. 2004), pregnant and non-pregnant rat myometrium (Noble et al. 2009), and human myometrial cells (Shlykov et al. 2003).

Electrophysiological evidence of SOCE has been primarily achieved by use of non-excitable tissue, as excitable cells have multiple ion channels, including L-type Ca^{2+} channels, with much greater conductance, and so it is difficult to isolate smaller currents, such as SOCE currents. Based primarily on evidence from non-excitable cells, a number of currents have been identified in response to Ca^{2+} depletion, describing a heterogeneous Ca^{2+} entry pathway. The first to be identified and that principally studied is I_{CRAC} (Ca^{2+} release activated Ca^{2+} current (Hoth et al. 1992). I_{CRAC} is a non-voltage dependent, highly Ca^{2+} selective (Hoth 1995; Parekh et al. 2005) inwardly rectifying current (Hoth et al. 1992). With a low single channel conductance of around 0.02pS (Parekh et al. 2005) and has been identified in many cell types.

Less is known about other currents induced by depletion of the store; I_{soc} (store operated currents) are relatively non-selective inwardly rectifying cation currents, often more permeable to Na^+ than Ca^{2+}, with generally higher single channel conductance than that of $I_{CRAC,}$ around 3pS (Trepakova et al. 2000; Trepakova et al. 2001; Albert et al. 2003a). And have been identified in a number of smooth muscles; rabbit portal vein (Albert et al. 2002),

aortic myocytes (Trepakova et al. 2001), and pulmonary artery myocytes (Golovina et al. 2001).

The molecular basis for SOCE in uterine smooth muscle has yet to be identified, but theories are centred on a number of proteins thought to make up the channel or channels required for SOCE, as functional analysis suggests a channel rather than an ion transporter is involved (Zweifach et al. 1993). The proteins thought to make these channels have been suggested to be; TRPC, Orai and STIM1.

The functional relevance of SOCE in uterine smooth muscle, is still relatively unknown. Myometrium has a large reliance of L-type VOCC, so the need for SOCE may be minor under normal physiological conditions. The myometrial capability of SOCE is upregulated in pregnancy and labour (Tribe et al. 2000; Noble et al. 2009), and so has been suggested to be utilised by agonists during labour. Oxytocin, a principal regulator of myometrial contractility, has been shown in both human and rat to elicit a SOCE-like current and shows the sustained increase in $[Ca^{2+}]_i$, which is sensitive to La^{3+} taken by many to be evidence of SOCE (Shimamura et al. 1994; Monga et al. 1999; Fu et al. 2000).

1.2.2 Potassium channels

There are a number of K^+ channels found in the uterus, contributing to repolarisation of the action potential and setting the resting membrane potential. Modulation of K^+ channels occurs through gestation and are thought to be responsible for control of uterine excitability, thus maintaining uterine quiescence during pregnancy. According to some

observations agonists can alter the function of K^+ channels. Multiple K^+ channels have been identified in the myometrium; Ca^{2+}-sensitive K^+ channel family, delayed rectifier, A-like and ATP-sensitive K^+ channels.

1.2.2.1 Ca^{2+}-sensitive K^+ channels

There are a number of functional K^+ channels found in uterine myocytes, although their complete role and mechanisms of function are still incompletely understood. Perhaps the most well studied of the K^+ channels is the large conductance K^+ channel (BK_{Ca}) also known as the maxi-K channel. BK_{Ca} are large conductance, voltage- and Ca^{2+}-sensitive K^+ channels, which were first described in smooth muscle in 1986 (Benham et al. 1986). A number of splice variants have been indentified, differing in their sensitivities to Ca^{2+}, voltage, hormonal sensitivity and ability to be phosphorylated (Brainard et al. 2007). They consist of four α-subunits that create a functional pore, with four modulatory β-subunits completing the protein. BK_{Ca} channels generally have a low affinity to Ca^{2+} and thus due to their low Ca^{2+} sensitivity require high levels of Ca^{2+} around BK_{Ca} channels for their activation (Neher 1998; Perez et al. 2001).

BK_{Ca} channels alter the excitability of the uterus, and so are modulated throughout gestation, allowing the uterus to be in a quiescent state during gestation and in a more excitable state during labour. At the end of gestation BK_{Ca} isoforms with lower sensitivity to Ca^{2+} (Korovkina et al. 2001; Benkusky et al. 2002; Curley et al. 2004), and those more sensitive to phosphorylation increase (Zhu et al. 2005). Thus decreasing the functional role of BK_{Ca} channels at the end of gestation and during labour (Wang et al. 1998), allowing the

uterus to be in a more excitable state during labour. A number of endogenous and exogenous ligands are able to exert their effects through BK_{Ca} channels. BK_{Ca} channels mediate uterine relaxation in response to adenylyl cyclase (Okawa et al. 2000) and to nitric oxide (Okawa et al. 1999). Meanwhile relaxin, generated by the feto-placental membranes and ovaries during gestation increase the open probability state of BK_{Ca} channels (Meera et al. 1995).

In addition to BK_{Ca} channels, two other Ca^{2+} sensitive K^+ channels have been identified in myometrial tissue; SK channel (small conductance Ca^{2+}-sensitive voltage insensitive K^+ channel (minK)) and IK channel (intermediate K^+ channels), both like BK_{Ca} channels are involved in maintaining the resting membrane potential and the generation of a hyperpolarising current following the generation of an action potential (Noble et al. 2009; Noble et al. 2010). Three SK isoforms have been identified (SK1-3), produced by three genes (KCNN1-3) (Kohler et al. 1996). They are all voltage independent and activated by Ca^{2+} at around 1 µM, and have a small single channel conductance of between 4-14 pS (Wray et al. 2010). There is evidence to suggest that Ca^{2+} release from the SR and Ca^{2+} entry from the extracellular space activate SK channels. Ca^{2+} binds to calmodulin, which is constitutively bound to the COOH terminus of the channel (Maylie et al. 2004) binding of Ca^{2+} leads to the opening of the channel.

Functionally SK3 decreases influx of Ca^{2+} through VOCC (Brown et al. 2007) and overexpression of SK3 results in inefficient contractions for labour (Bond et al. 2000) suggesting that the membrane potential is in a hyperpolarised state, maintaining the block on labour. SK3 channels are also involved in relaxation induced by nitric oxide (Modzelewska et al. 2003a; Modzelewska et al. 2003b).

Whilst knowledge of SK channels is limited, information regarding IK channels is virtually non-existent. IK channels, formally SK4, have a 40 % αα homology to SK1-3 (Begenisich et al. 2004). Have a conductance of 50-70 pS (Vandorpe et al. 1998), and unlike SK channels are insensitive to apamin and are not voltage dependent.

1.2.2.2 Voltage-gated K^+ channels

Voltage-gated K^+ channels (Kv channels) are essential for the control of spontaneous activity of myometrial smooth muscle (Aaronson et al. 2006). They are activated by depolarisation, are responsible for repolarisation of the membrane following the depolarisation phase of the action potential, and are also involved in maintaining resting membrane potential, contributing to the maintenance of uterine quiescent before labour (Lundgren et al. 1997).

Kv channels constitute a heterogeneous family, giving numerous voltage-gated K^+ currents, which differ in their kinetics and sensitivity to inhibitors (Brainard et al. 2007). In uterine smooth muscle, two types of Kv current have been identified; delayed rectifiers and rapidly inactivating currents (Wang et al. 1998; Knock et al. 1999; Knock et al. 2001). The principal voltage-gated K^+ current identified in pregnant rat myometrium is a rapidly inactivating K^+ current, generated by the Kv4 subfamily (Suzuki et al. 2005). Members of the Kv4 subfamily are known to be modulated by oestrogen and 17β-estradiol, increasing expression and current through these channels at parturition (Knock et al. 2001; Song et al. 2001).

1.2.2.3 ATP-sensitive K^+ channels

The ATP-sensitive K^+ channel (K_{ATP}), constitutes an inwardly rectifying K^+ channel, which has a role in the regulation of myometrial quiescent during pregnancy. They consist of Kir6

subunit forming the K^+ channel, while the sulfonylurea receptor (SUR) confers ATP and other pharmacological sensitivities (Inagaki et al. 1996; Clement et al. 1997; Babenko et al. 1999). ATP inhibits the channel while MgADP stimulates, thus coupling cellular excitability to the metabolic state of the cell (Dunne et al. 1986), and so may provide the mechanistic link in dystocia, which has links to hypoxia (Heaton et al. 1993).

Similar to other K^+ channels, studies have suggested that the predominant isoform Kir6.1/SUR2B is downregulated towards parturition (Curley et al. 2002). K_{ATP} channels mediate relaxation in response to adenylyl cyclase and guanylyl cyclase (Okawa et al. 2000).

1.2.3 Ca^{2+} activated Cl^- channels

Ca^{2+}-activated Cl^- (Cl_{Ca}) channels have been found in the uterus (Coleman et al. 1987), with ICl_{Ca} found in 30% of pregnant rat myocytes (Jones et al. 2004). They constitute a Cl^- efflux pathway, activated by Ca^{2+}entering the cell via L-type Ca^{2+} VOCC or release from the SR (Jones et al. 2004), resulting in the generation of an inward current, and depolarisation of the cell membrane. Cl_{Ca} channels are responsible for transient inward currents in smooth muscles (Wray et al. 2010), although there is currently no evidence for either Ca^{2+} sparks or STICs in the myometrium. But they do play a role in spontaneous phasic contractility; with inhibition reducing frequency and duration of contractions (Jones et al. 2004; Young et al. 2009). They also play an important role in response to agonists, being activated by the increase in Ca^{2+}, released from the SR in response to IP_3 (Arnaudeau et al. 1994).

1.2.4 Ca^{2+} removal mechanisms

Myometrial contraction is Ca^{2+}-calmodulin dependent, and as such relaxation is dependent on a fall in $[Ca^{2+}]_i$. In resting cells there is a 10,000-fold concentration gradient across the plasma membrane, in order to maintain this steep concentration gradient and control the reduction in $[Ca^{2+}]_i$ following influx through L-type VOCC, three Ca^{2+} removal mechanisms exist; two Ca^{2+} extrusion systems which include the plasma membrane Ca^{2+}-ATPase (PMCA) and the Na^+/Ca^{2+} exchanger (NCX) and two systems of intracellular Ca^{2+} sequestration which include the SERCA pump of the sarcoplasmic reticulum (SR) and the Ca^{2+} uniporter of the mitochondria. Functionally PMCA along with NCX is critical in the efflux of Ca^{2+}, with the PMCA being responsible for the extrusion of around 70% of total Ca^{2+} efflux (Shmigol et al. 1998a). There is also a correlation with an increase in PMCA expression and the onset of labour (Tribe et al. 2000).

1.2.4.1 PMCA

The PMCA is a member of the P-type ATPase family, as such the pump uses hydrolysis of ATP to overcome the concentration gradient of Ca^{2+}, counter-transporting Ca^{2+} against H^+, at a stoichiometery of one Ca^{2+} to one molecule of ATP hydrolysed (Wuytack et al. 1992). It is encoded by four genes, and alternative splicing gives rise to twenty-six isoforms, primarily with different regulatory properties (Carafoli 2004). Isoforms are composed of ten transmembrane spanning domains, primarily responsible for the catalytic domain and a long C- terminus regulatory domain (Monteith et al. 1995), and are localised within caveolae (Fujimoto 1993).

In its inactive state, PMCA is autoinhibited by its calmodulin binding domain within the regulatory domain, in a similar fashion to that found for myosin light chain kinase (MLCK), having a low affinity for Ca^{2+} (Verma et al. 1994). Upon binding calmodulin, auto-inhibition is removed, increasing both Ca^{2+} affinity and maximum velocity of the pump activity (Roufogalis et al. 1980; Muallem et al. 1981).The PMCA can also be activated by acidic phospholipids such as PIP_2, protein kinases most commonly PKA and PKC, with the degree of activation and modulatory mechanism dependent on isoform expression (Monteith et al. 1995; Penniston et al. 1998). Critically both G-proteins and agonists have been shown to activate and modulate PMCA, allowing another mechanism in which they can increase myometrial activity (Monteith et al. 1995).

1.2.4.2 Na^+/Ca^{2+} exchanger

The Na^+/Ca^{2+} (NCX) exchanger is an electrogenic antiporter, using the Na^+ gradient maintained by the Na^+, K^+-ATPase to drive the extrusion of one Ca^{2+} for three Na^+. In physiological condition the NCX will act to extrude Ca^{2+}, but can reverse depending on the prevailing concentrations of intra- and extracellular Na^+ and membrane potential (Aickin et al. 1984; Carafoli 2004). Compared to the PMCA, NXC has a lower affinity for Ca^{2+} but a higher capacity system, so it is suggested that NCX has a role at higher $[Ca^{2+}]_i$, rather than fine tuning responses which is more suited to the PMCA (Matthew et al. 2004). While the PMCA has been shown to be responsible for around 70% of Ca^{2+} efflux, the NCX is responsible for the remaining 30% (Shmigol et al. 1998a). As with many proteins, there is a gestational change in the reliance of the NCX, having a greater role in Ca^{2+} extrusion in response to agonists in late pregnancy (Taggart et al. 1997).

Three genes (NCX 1-3) and alternative splicing are responsible for the eight NCX family members, giving a 938 amino acid protein containing nine transmembrane domains, with a large cytosolic loop between transmembrane domains 5-6 thought to contain the regulatory domains; including biding sites for calmodulin (Carafoli 2004). Similarly to PMCA, they are also localised to caveolae in smooth muscle (Moore et al. 1993; Juhaszova et al. 1994).

1.2.4.3 Na^+/ K^+-ATPase

Normal functioning of myometrial smooth muscle is dependent on maintenance of the correct ionic balance across the membrane. High intracellular K^+ and low Na^+ concentrations require the active transport of both ions across the membrane, which is achieved by the Na^+, K^+-ATPase. The Na^+, K^+-ATPase, like the PMCA and SERCA is a member of the P-type ATPase, and so hydrolysis of ATP provides the energy to counter transport two K^+ and three Na^+ against their concentration gradients (Floyd et al. 2007).

Na^+, K^+-ATPase is composed of three subunits; α subunit responsible for catalysis and transport of ions, β subunit which modulates the α subunit, and μ subunit which is also involved in modulation of Na^+, K^+-ATPase activity.(Floyd et al. 2007; Floyd et al. 2010). There are multiple isoforms of each subunit, expression been both species and tissue specific, these isoforms have different sensitivities and regulation by $[Na^+]_i$, $[Ca^{2+}]_i$, PKA and PKC (Decollogne et al. 1993). In uterine smooth muscle, there is a switch in isoform expression in pregnancy, although the functional significance of this is unknown (Floyd et al. 2010).

1.3 The sarcoplasmic reticulum

Experiments in Ca^{2+} free solutions, showing that contractile responses continued in the absence of external Ca^{2+}, first identified a role for a smooth muscle intracellular Ca^{2+} store (Hurwitz et al. 1967). The sarcoplasmic reticulum (SR) was first suggested to be the intracellular Ca^{2+} store in smooth muscles in 1971 based on EM studies (Gabella 1972) and the ability of Sr^{3+} to accumulate in the SR (Somlyo et al. 1971). Since then it has been established that the SR plays a pivotal role in agonist induced contractility in myometrial smooth muscle (Shmygol et al. 2005).

1.3.1 Ca^{2+} release

1.3.1.1 Inositol 1,4,5-triphosphate receptors

Inositol 1,4,5-triphosphate receptors (IP_3R) are one of two SR Ca^{2+} release pathways, found in smooth muscle. Due to the non-functional ryanodine receptors (RyR), it is the primary release mechanism found in uterine smooth muscle. They release Ca^{2+} from the SR in response to agonist stimulation. Binding of G-protein coupled receptors (GPCR), activates phospholipase C (PLC), leading to the hydrolysis of phosphatidylinositol 4,5-biphosphate (PIP_2) yielding diacylglycerol (DAG) and inositol 1,4,5-tripphosphate (IP_3) (Berridge et al. 1984). IP_3 directly activates IP_3R via the N-terminal ligand binding domain allowing the efflux of Ca^{2+} (Yoshikawa et al. 1996).

IP_3R exist as tetrameric structures, having a monomeric molecular mass of around 300 KDa and can consist of both homo- and heteromeric assemblies (Devogelaere et al. 2008). Three distinct IP_3R gene products (1-3) have been identified, all three are structurally and functionally similar (Mikoshiba et al. 1994), although expression subtype has not to date been determined for the myometrium.

In addition to being activated by IP_3, there are a number of intracellular modulatory mechanisms, the principal being $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ has a biphasic action on the effects of Ca^{2+} release by IP_3R; at low concentrations IP_3R are stimulated and so require less IP_3 to give a bigger Ca^{2+} release, while at higher $[Ca^{2+}]_i$ IP_3R are suppressed, needing more IP_3 to give the same Ca^{2+} release (Patel et al. 1999; Wray et al. 2010).

1.3.1.2 Ryanodine receptors

RyR are the other Ca^{2+} release mechanisms found in smooth muscle, named after the plant alkaloid which it binds. RyR are homotetramers forming a central pore, allowing the co-ordinated movement of Ca^{2+} through the pore, with each subunit associated with a regulatory binding protein FKBP (Meissner 2004). They have a large cytosolic domain, containing binding sites for Ca^{2+}, calmodulin in addition to phosphorylation sites (Wray et al. 2010), allowing for the modulation of Ca^{2+} flux. The principal endogenous activator is Ca^{2+}, and similar to IP_3R, Ca^{2+} has a biphasic response, activating RyR at low concentrations, whilst inactivating at higher cytosolic concentrations (Meissner 1994).

There are three isoforms of RyR (1-3), encoded by distinct genes, having variable pharmacological and kinetic profiles (Sutko et al. 1997). All three isoforms can be expressed

in smooth muscle, including rat myometrium, with RyR3 being the dominant isoform (Martin et al. 1999). Although RyR are expressed in rat myometrium, Ca^{2+} sparks have not been identified, and so RyR are considered to be non-functional (Burdyga et al. 2007).

1.3.1.3 Ca^{2+} leak

There is a low level constitutive non-regulated Ca^{2+} leak through the SR membrane; shown by inhibiting Ca^{2+} channels and preventing Ca^{2+} reuptake to the store by SERCA. The nature of this Ca^{2+} leak is not understood, but has been suggested to be needed to balance SERCA activity (Camello et al. 2002). In uterine smooth muscle, little investigation of Ca^{2+} leak has taken place, although in human uterus little depletion occurred with inhibition of SERCA (Young et al. 1999).

1.3.2 Ca^{2+} uptake

1.3.2.1 Sarco/endoplasmic reticulum Ca^{2+}-ATPase

The sarco / endoplasmic reticulum Ca^{2+}-ATPase (SERCA) is a P-type ATPase responsible for the uptake of Ca^{2+} into the SR. Similar to the PMCA and Na^{+},K^{+}-ATPase, it utilises the energy from ATP hydrolysis, to transport two Ca^{2+} into the SR for every ATP hydrolysed, and so acts to maintain the store.

The pump was first purified in 1970 (MacLennan 1970), with the mechanism of action elucidated in 2002 (Toyoshima et al. 2002). Two Ca^{2+} from the cytosolic side bind, and upon phosphorylation of Asp351 by ATP, Ca^{2+} is translocated across the membrane by the high energy intermediary and so becomes a low energy intermediary releasing Ca^{2+} into the SR lumen (for review see Wray et al. 2010). During this cycle two or three protons are counter transported ensuring partial charge balance (Levy et al. 1990).

There are 3 mammalian isoforms, encoded by three genes (ATP2A1, ATP2A2, ATP2A3), known as SERCA-1, -2, and -3. In addition, alternative splicing occurs, giving rise to SERCA1-a and –b, and SERCA2 – a and -b. SERCA2 is found in smooth muscles, with a greater expression of SERCA2b in the uterus (Khan et al. 1993), while both are expressed to a greater extent in pregnant compared to non-pregnant and in labouring samples (Khan et al. 1993; Tribe et al. 2000). All isoforms are structurally similar, and are composed of three parts; a large cytosolic head, constituting half of SERCA, a stalk domain and a 10 transmembrane spanning domain (Wray et al. 2010).

The principal regulator of SERCA is phospholamban, when bound it inhibits SERCA by lowing its apparent affinity for Ca^{2+} through direct protein-protein interactions (Asahi et al. 2003), but is phosphorylated by both PKA and Ca^{2+}-calmodulin-dependent protein kinase II, resulting in reduction in SERCA inhibition (Simmerman et al. 1998). SERCA activity is also modulated by a number of other regulators including insulin-receptor substrate (IRS1/2), Ca^{2+} binding protein S100A1 and Bcl-2 (Wray et al. 2010). It is also regulated indirectly by Ca^{2+} through the protein calreticulin, when luminal $[Ca^{2+}]_i$ are low calreticulin does not bind to SERCA, removing inhibition, therefore increasing SERCA activity, increasing the ability to fill the store (Michalak et al. 1999).

1.4 Excitation – contraction coupling

The uterus is a myogenic organ, that is, contractions can be initiated intrinsically by the myocyte, without the involvement of an external stimulus, such as nervous or hormonal inputs. In uterine smooth muscle, contraction is Ca^{2+}-dependent, with an increase in $[Ca^{2+}]_i$ needed for contraction to occur, this increase in $[Ca^{2+}]_i$ is the result of Ca^{2+} influx through L-type VOCC as part of the action potential, or by other mechanism such as store operated Ca^{2+} entry and receptor operated Ca^{2+} entry. In smooth muscle excitation-contraction coupling can occur through two mechanisms; firstly by electro-mechanical coupling, a term coined to describe the relationship between membrane potential and the induction of mechanical force, explaining the relationship between action potential and force production (Somlyo et al. 1994). The second mechanism is by pharmaco-mechanical coupling, whereby stimuli result in an increase in the production of force that is not dependent on changes in membrane potential, although changes in membrane potential may occur, examples of this are Ca^{2+} entry through ROC, SOCE and Ca^{2+} sensitisation (Somlyo et al. 1994).

1.4.1 Electro-mechanical coupling

As previously described, electro-mechanical coupling is the relationship between membrane potential and the mechanical output of a muscle. This relationship is pivotal to uterine smooth muscle contraction, both spontaneous and agonist induced contractility is dependent upon depolarisation of the membrane resulting activation of an action potential, with a resulting influx of Ca^{2+}through L-type VOCC. Changes in membrane potential by agonists and other modulators of uterine contractility, will alter the frequency of action

potentials and therefore force characteristics. They are able to do this by working on ion channels, such as BK_{Ca} and Cl_{Ca}, as previously discussed.

1.4.1.1 The action potential

In smooth muscle there are two forms of action potential; spike-like and plateau. Rat longitudinal myometrial tissue, unlike other types of smooth muscle, exhibit spike-like action potentials and do not possess plateau-like action potentials found in other species, and so only spike-like action potentials found in rat myometrial tissue will be discussed (Mironneau 1973; Kuriyama et al. 1976; Parkington et al. 1988).

Unlike other smooth muscle, the mechanism behind the initiation of action potentials in uterine smooth muscle have yet to be identified. In other smooth muscle, pacemaker cells have been identified, they have an unstable fluctuating membrane potential. When this membrane potential reaches threshold activation of VOCC, an action potential is initiated. Whilst pacemaker cells have not been identified in the uterus, slow rhythmic alterations in membrane potential have been detected, although the underlying ionic mechanism has yet to be elucidated (Casteels et al. 1965; Sanborn 1995).

Once the slow wave of depolarisation reaches the threshold activation of L-type VOCC, around -40mV (Jmari et al. 1986) channels open and there is an influx of Ca^{2+}, this in conjunction with an increase in Na^{+} permeability results in the upstroke of the action potential (Shmigol et al. 1998b). The repolarisation phase, downstroke of the action potential is due to a decrease in the inward Ca^{2+} and Na^{+} currents and the development of an outward current (Mironneau et al. 1980; Mironneau et al. 1981). L-type VOCC as

discussed previously, are voltage sensitive, and upon depolarisation are inactivated (Honore et al. 1989; Sperelakis et al. 1992), this in addition to time- and Ca^{2+}- dependent inactivation of L-type VOCC will drastically reduce Ca^{2+} influx, whilst voltage and Ca^{2+}-dependent K^{+} channels are activated resulting in the repolarisation of the cell (Mironneau et al. 1980; Mironneau et al. 1981).

1.4.1.2 Relationship between action potentials, Ca^{2+} spikes and force

Unlike other forms of smooth muscle, rat myometrial tissue only exhibit spike like action potentials, with force characteristics dependent upon the characteristics of the bursts of action potentials. Each action potential correlates to an individual Ca^{2+} spike, and a transient increase in force (Burdyga et al. 2009). The higher the frequency of action potentials with a burst of action potentials, increases the frequency of concurrent Ca^{2+} spikes, resulting in a higher production of force, as the result of summation (Mironneau 1973; Burdyga et al. 2009) (Figure 1.4.1.2).

In addition to action potentials being responsible for the amount of force produced, they are also responsible for the frequency and duration of contractions. Myometrial contractions are Ca^{2+}-dependent, and as such are activated by influx through L-type VOCC as part of the action potential, and so contraction frequency is dependent upon the frequency of the bursts of action potentials. While duration will remain for the length of the action potential burst (Mironneau 1973), with rate of relaxation also dependent upon the rate of MLC dephosphorylation.

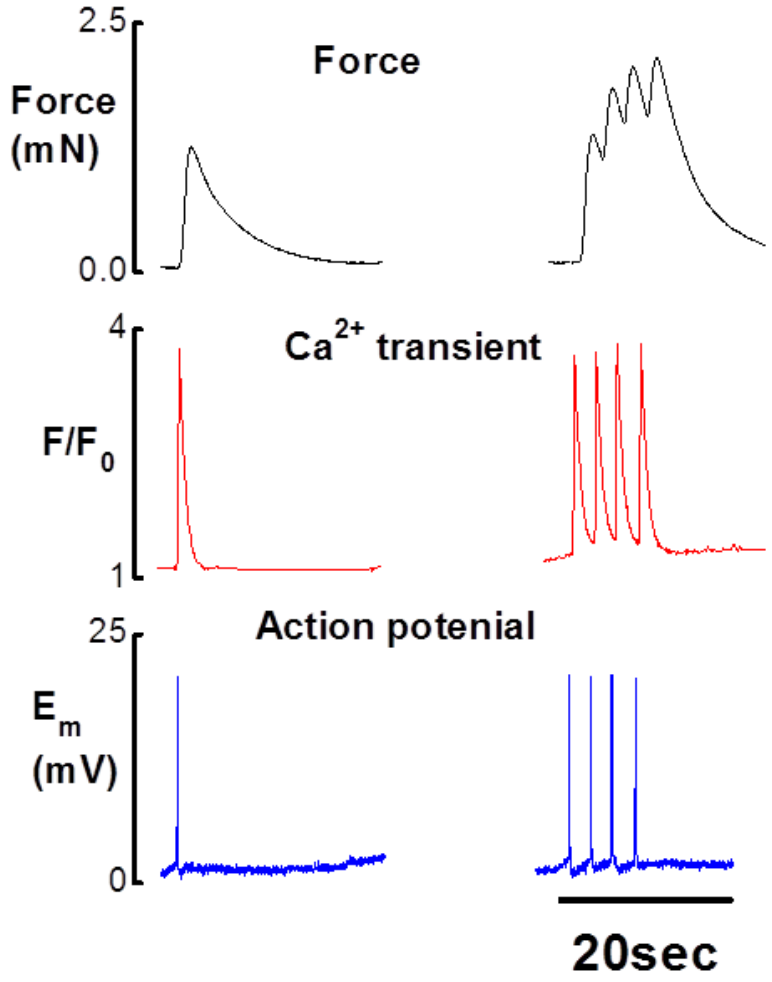

Figure 1.4.1.2 The relationship between action potentials, Ca^{2+} spikes and force generation. Original trace of electrical activity (blue), $[Ca^{2+}]_i$ (red), and force (black). A single action potential correlates to an individual Ca^{2+} spike, and a transient increase in force, higher frequency of action potentials, increases the frequency of Ca^{2+} spike, and force increases due to summation. (Burdgya T et al. 2009)

1.4.1.3 Propagation of action potentials

In order for the uterus to function, it needs to provide phasic contractions of the appropriate duration, frequency and force. To do this, action potentials must be appropriately propagated throughout the tissue, allowing synchronisation of the longitudinal muscle bundles. The ability of myometrial tissue to initiate and propagate action potentials, is constantly changing. The initiation site can move, while the speed, direction and length of propagation can alter within minutes (Lammers et al. 1994; Lammers et al. 1998). It is limitations in the spatial spread of action potentials that maintain uterine quiescence during gestation, with an increase in the propagation, velocity and conduction of action potentials increasing towards parturition, giving the synchronous activity needed to expel the foetus (Cole et al. 1985; Lammers et al. 1994; Doring et al. 2006).

Intracellular communication is thought to be provided by gap junctions. Gap junctions are specialised conduits that allow direct intracellular communication by the passage of molecules of up to 1000 Da, directly coupling both electrical and metabolic states of individual cells. They are modulated throughout gestation, with increasing density at the end of gestation, correlating with the increase in electrical coupling (Cole et al. 1985; Lammers et al. 1994); while gap junction density is minimal during gestation, explaining the lack of propagation and conduction of action potentials during pregnancy. The importance of gap junctions in contractility is highlighted by inhibition of gap junctions, which results in delayed parturition (Cole et al. 1985; Lammers et al. 1994; Doring et al. 2006).

1.4.2 Pharmaco-mechanical coupling

Pharmaco-mechanical coupling refers to the production of force, which is independent of changes in the membrane potential, often induced by endogenous and exogenous agonist. In uterine smooth muscle, this can occur through a number of mechanisms, such as; Ca^{2+} entry through ROC/NSCC, SOCC and release from the SR, it may also result from Ca^{2+}-sensitisation. Whilst pharmaco-mechanical coupling is not initiated by a change in membrane potential, it is important to note that some pharmaco-mechanical coupling mechanisms may have downstream effects on membrane potential, and dependent processes.

1.4.2.1 Ca^{2+} entry

The binding of an agonist to its receptor can result in the influx of both Ca^{2+} and other ions into the cell, increasing $[Ca^{2+}]_i$, resulting in contractility. They are able to do this by two mechanism; receptor-operated Ca^{2+} entry (ROCE) of store-operated Ca^{2+} entry (SOCE). ROCE is dependent upon the activation of a heterogeneous pool of either Ca^{2+} or non-specific channels as a result of agonist binding, or its downstream effects (as previously discussed). While SOCE, is activated in response to agonist depletion of the SR, and similar to ROCE, has been identified as involving both Ca^{2+} and non-selective currents (as previously discussed). In addition to increasing $[Ca^{2+}]_i$, changes in the conductance of either Ca^{2+} or other ions as a result of either ROCE or SOCE will effect membrane potential and thus modulate L-type VOCC, contributing to the response of agonists. (Refer to ROC or SOCE for detailed discussion)

1.4.2.2 SR Ca^{2+} release

In addition to the activation of ROCE, agonists often release Ca^{2+} from the SR. Binding of G-protein coupled receptor (GPCR) results in the activation of PLC, increasing IP_3, IP_3 binds to IP_3R on the SR, releasing Ca^{2+}, increasing $[Ca^{2+}]_i$ (See IP_3R for more detail). The release of Ca^{2+} from the SR, often results in depletion of the store, and activation of SOCE, further enhancing $[Ca^{2+}]_i$.

1.4.2.3 Ca^{2+} sensitivity

Agonists are able to modulate the Ca^{2+} sensitivity of the contractile machinery, and so smooth muscles are able to produce more force, for the same $[Ca^{2+}]_i$. There is evidence of a number of uterotonics working through Ca^{2+} sensitisation; including oxytocin (Izumi et al. 1996). Although evidence of Ca^{2+} sensitisation in the myometrium is limited, evidence of its functional importance and its mechanisms are more prevalent in other smooth muscles, with Ca^{2+} sensitisation especially important to the creation of tone in vascular smooth muscle (Pfitzer 2001). The primary mechanisms by which modulation of Ca^{2+} sensitisation occurs is by Ca^{2+}-independent modulation of myosin light chain kinase (MLCK) and or myosin light chain phosphotase (MLCP), primarily through PKC and rho-kinase, thus altering the proportion of MLC_{20}-P for a given $[Ca^{2+}]_i$. (See MLCK and MLCP for more detailed discussion).

1.5 Mechanism of contraction

1.5.1 Basis of muscle contraction

Uterine smooth muscle contraction is Ca^{2+}-calmodulin-myosin light chain kinase dependent, with multiple lines of evidence showing that removal of this pathway results in the abolishment of contractility (Longbottom et al. 2000). During stimulation, whether that be spontaneous or agonists induced, $[Ca^{2+}]_i$ rise from around 10^{-7} to 10^{-5}. Ca^{2+} binds to and activates calmodulin. Ca^{2+}-calmodulin has two pivotal functions in smooth muscle contractility; firstly it activates myosin light chain kinase (MLCK), the enzyme responsible for the phosphorylation of MLC_{20}. Phosphorylated-MLC_{20} (MLC_{20}-P) activates the myosin ATPase, allowing myosin to interact with actin as part cross-bridge cycling and so force is generated (Figure 1.5.1) (Word 1995) (Figure 1.5.1). The second function of Ca^{2+}-calmodulin is its interaction with caldesmon, upon binding caldesmon inhibition of actin-myosin interactions is abolished, and force can be produced (Wang 2001).

Upon phosphorylation of MLC_{20} and removal of caldesmon block, actin thin filaments and myosin are able to interact in cross-bridge cycling and the generation of force. This is the same biochemical mechanism that occurs in striated muscle. Briefly by hydrolysis of ATP, the myosin head undergoes a conformational change, pulling the actin thin filaments, resulting in contraction. The cycle occurs in four steps; firstly myosin lacking nucleotide binding is tightly bound to actin, upon ATP binding to myosin, there is a decrease in affinity for actin, and thin and thick filaments are no longer attached. Hydrolysis of ATP results in a conformational change that causes the myosin head to become primed. Weak interactions between actin and myosin result in the release of ADP and inorganic phosphate (Pi), increasing the interaction and triggering the power-stroke, were the myosin head regains its

original shape, pulling the thin filaments along the myosin, which returns the actin and myosin to the beginning of the cycle.

Relaxation of smooth muscle occurs when $[Ca^{2+}]_i$ is restored to resting levels, as a result of efflux from the cell by PMCA and NCX or the sequestering of Ca^{2+} into the SR by SERCA (Matthew et al. 2004). The decrease in $[Ca^{2+}]_i$ results in a reduction in Ca^{2+}-calmodulin, which can therefore no longer activate MLCK, while dephosphorylation of MLC_{20} still occurs via MLCP. This shift in the balance between phosphorylation and dephosphorylation of MLC_{20} results in the relaxation of the muscle.

While this pathway is essential to contraction, modulation of many steps occurs in the fine tuning of muscle contractions. This primarily occurs through MLCP, MLCK and the thin filaments.

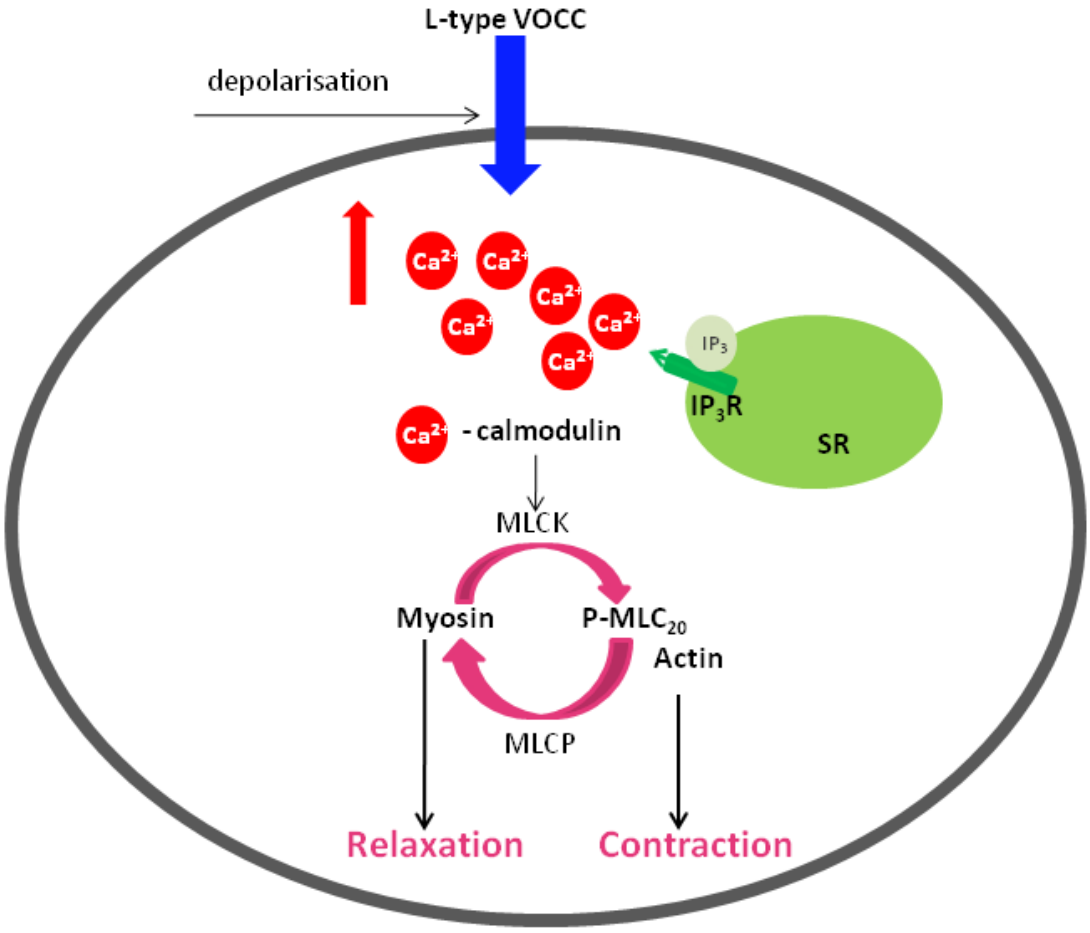

Figure 1.5.1 A basic scheme to show how an increase in $[Ca^{2+}]_i$ leads to myometrial contraction. An influx of Ca^{2+} through L-type VOCC in addition to SR Ca^{2+} release, increases $[Ca^{2+}]_i$, Ca^{2+} binds and activates calmodulin, Ca^{2+}-calmodulin can then activate MLCK and subsequent phosphorylation of MLC_{20}, leading to cross-bridge cycling. Relaxation is dependent upon MLC_{20} dephosphorylation by MLCP.

1.5.2 Contractile proteins

Uterine, similar to other smooth muscles, are composed of two major contractile proteins; thick and thin filaments, with thin filaments composed of actin and tropomyosin along with the regulatory proteins caldesmon and calponin (Hodgkinson 2000). Smooth muscle generate force with cross-bridge cycling as found in striated muscle, and contains generally the same contractile apparatus, with a few obvious exceptions; troponin is not expressed in smooth muscle, and the addition of the regulatory protein caldesmon, calponin and calmodulin. The structural organisation is very different, while striated muscles are composed of highly structured sarcomeres, smooth muscle is less organised, and maintained by dense bodies (Ashton et al. 1975; Ali et al. 2005)(See figure 1.5.2 for schematic diagram of contractile proteins and organisation).

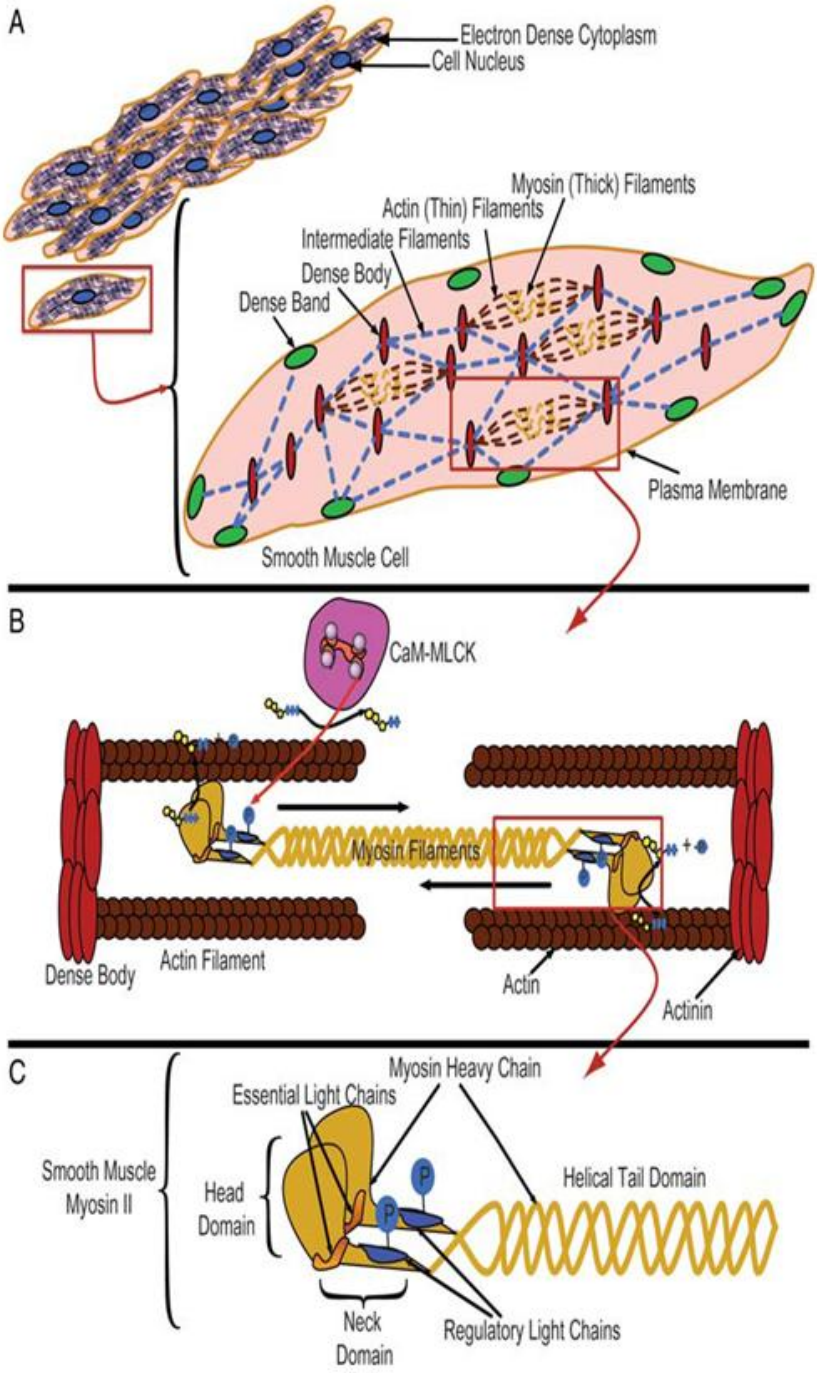

Figure 1.5.2 Schematic overview of A) contractile apparatus in a myocyte, B) thick and think filament organisation, D) structure of myosin. (Aquilar & Mitchell, 2010)

1.5.2.1 Myosin (thick filaments)

Myosin comprises a large family of ATPases, who in conjunction with actin are responsible for the movement during cross-bridge cycling. Myosin II is responsible for the contraction of muscle, and there are multiple homologues, having the same basic structure; hexamers composed of two myosin heavy chains (230KDa) and two pairs of myosin light chains (MLC) (17 and 20KDa). The two heavy chains form an α-helical coiled-coiled structure that includes the sites of myosin molecule association for filament formation, and extends through the hinge region (converter domain), responsible for the movement of the globular head, into the globular head, which is the site of actin binding and ATP hydrolysis (Eddinger et al. 2007). The MLCs are associated with the globular head region of the heavy chain, were MLC_{20} is thought to inhibit the actin-activated ATPase, upon phosphorylation, at either Ser19 or Thr18, there is a conformational change and this inhibition is removed (Ikebe et al. 1983; Ikebe et al. 1988).

1.5.2.2 Thin filaments

While myosin phosphorylation is the determining factor in smooth muscle contractility, it is the smooth muscle thin filaments that are thought to provide the fine tuning regulation required for effective contractility. Smooth muscle thin filaments are composed of actin, tropomyosin, caldesmon and calmodulin in ratios of 14:2:1:1. All four components are needed for correct cross-bridge cycling and its regulation.

The principal component of thin filaments is actin. In smooth muscle, actin is made up of smooth muscle α-actin (ACTA2 gene) and γ-actin (ACTAG2 gene), with proportions

dependent upon the smooth muscle (Marston et al. 2008). Actin is a highly conserved protein with a molecular mass of 42 KDa. With all actin monomers (globular G-actin) able to polymerise and form fibrillar F-actin macromolecules, forming a right handed helix composed of two strands that cross every 36 nm, containing 13 G-actin (Hodgkinson et al. 1997a; Hodgkinson et al. 1997b; Hodgkinson 2000). Each G-actin has a high-affinity myosin head binding site.

Tropomyosin is the second most abundant thin filament protein. There are two smooth muscle isoforms α- and β- tropomyocin, produced by alternative splicing. They are approximately 284 amino acids in length, existing as heterodimers in a predominantly α-helical coiled-coiled structure, wrapped around the α-helical actin strands (Jancso et al. 1991; Hodgkinson 2000).

Caldesmon is the third most abundant thin filament protein, after actin and tropomyocin. It is an elongated molecule of approximately 87 KDa, composed of three domains. The C-terminal region is considered to be the most important domain with primary binding sites for; actin, calmodulin and other phosphorylation sites (Wang 2001). While the N-terminal is also able to interact with actin and calmodulin, it can only do this weakly, it also contains a myosin binding domain (Wang 2001). The two regions are interspaced by a middle spanning region, composed of a highly charge repeating sequence with no clear function. It is this spanning region that gives the shape to caldesmon, appearing as a stretch dumbbell (Wang 2001).

Caldesmon acts to inhibit myosin ATPase activity (Ngai et al. 1984) acting as a break to myometrial contractility. In conjunction with its role as an inhibitor to myometrial contractility, expression is significantly increased in pregnancy (Word et al. 1993).It is

thought to act as a myometrial contractile inhibitor by tethering actin and myosin together and blocking their direct interaction. The N-terminal region of caldesmon binds myosin, while its C-terminal binds actin, in a low $[Ca^{2+}]_i$ /unstipulated state this configuration blocks the binding of unphosphorylated myosin heads to actin. Upon stimulation, Ca^{2+}-calmodulin interacts with caldesmon resulting in the movement of caldesmon away from actin/myosin interaction sites (Wang 2001). In addition to Ca^{2+}-calmodulin inhibition of caldesmon, it is also able to be phosphorylated in a non-Ca^{2+} dependent manner by PKC and protein kinase II (Gorenne et al. 2004).

Calponin is another regulatory protein associated with the thin filaments, and found in all smooth muscles. It is a monomeric protein of 34 KDa (Winder et al. 1993), with similar binding characteristic to caldesmon, able to interact with actin (Childs et al. 1992) myosin (Szymanski et al. 1997) and Ca^{2+}-calmodulin (Winder et al. 1993). Unlike caldesmon, it appears that the only physiological relevant interaction partner is actin (Winder et al. 1993). Calponin inhibits the myosin ATPase activity rather than reducing Ca^{2+}-dependent myosin phosphorylation (Winder et al. 1990) and is thought to involve inhibition of a catalytic step ATPase cycle, most probably the rate limiting step of ADP or inorganic phosphate (Pi) release.

Calponin itself is regulated by phosphorylation, resulting in the abolishment of calponin inhibition on the actin-activated ATPase (Winder et al. 1993). Both PKC and calmodulin kinase II have been shown to phosphorylate calponin (Winder et al. 1990) while dephosphorylated by a type 2A protein serine/threonine phosphatase (Winder et al. 1992).

1.5.3 Regulatory protein

1.5.3.1 Myosin light chain kinase

Myosin light chain kinase (MLCK) is a specific serine/threonine protein kinase, capable of phosphorylating only MLC_{20}, at either Ser19 or Thr18, activating cross-bridge cycling and thus the production of force (Moore et al. 2001; Takashima 2009). It is a monomer, consisting of; a catalytic domain, calmodulin regulatory domain, an actin-binding domain at its N-terminus and a myosin binding region at its C-terminal (Guerriero et al. 1986; Kohama et al. 1996; Stull et al. 1998; Kudryashov et al. 1999).

The catalytic domain is responsible for the phosphoylation of MLC_{20} at Ser19, at higher concentration MLCK will also phosphorylate Thr18 (Ikebe et al. 1986; Horowitz et al. 1996). At low $[Ca^{2+}]_i$ the autoinhibitory calmodulin regulatory domain intrasterically inhibits MLCK activity (Gallagher et al. 1993; Moore et al. 2001). Upon influx of Ca^{2+}, 3-4 Ca^{2+} bind to calmodulin, Ca^{2+}-calmodulin binds to the calmodulin binding domain of MLCK, resulting in a conformational shift and the removal of the autoinhibition of the catalytic subunit by the calmodulin binding domain, allowing the phosphorylation of MLC_{20} (Olson et al. 1990; Moore et al. 2001).

1.5.3.1.1 Modulation of MLCK

MLCK is the protein responsible for activation of MLC_{20}, and as such its activity is tightly regulated. To date less is known about the regulation of MLCK compared to the more interesting MLCP regulation, but it is known that regulation, similar to MLCP, is primarily dependent upon phosphorylation, resulting in a decrease in activity. MLCK can be phosphorylated by a number of modulators; primarily Ca^{2+}-calmodulin-activated protein kinase II, but also protein kinase A (PKA) and protein kinase C (PKC) (Moore et al. 2001). Phosphorylation of MLCK results in a reduction in affinity of MLCK for the Ca^{2+}-calmodulin complex, therefore reducing the amount of activated MLCK (Stull et al. 1998; Pfitzer 2001).

1.5.3.2 MLCP

Myosin light chain phosphatase is the only known endogenous phosphatase capable of dephosphorylating MLC_{20}, it therefore has a pivotal role in the regulation of myometrial contractility. MLCP is composed of three subunits; a 110- to 130 KDa regulatory subunit (MYPT1), a 37 KDa catalytic subunit (PP1c) and a 20 KDa subunit of unknown function (Hartshorne 1998).

For maximal functioning of the catalytic subunit, it needs to be bound to MYPT1. This is achieved though the PP1c binding domain located at the N-terminal of MYPT1 in addition to the ankyrin repeats (Alessi et al. 1992; Shirazi et al. 1994). The ankyrin repeats are a dominant feature of the N-terminal of the protein, as they are found in a variety of proteins, they are thought to confer many of the biding properties of MLCP acting as an appropriate platform for interactions (Hartshorne 1998). It is also likely that the ankyrin repeats are

required for binding P-MLC_{20} (Hirano et al. 1997), in addition to binding myosin and PP1c, MYPT1 also contains the phosphorylation sites for rho-kinase inhibition (Hartshorne 1998). While the regulatory subunit is needed for regulation and maximal function of the holoenzyme, it is the catalytic, PP1C subunit that is responsible for the dephosphorylation of P-MLC_{20}, while the function of the 20 KDa subunit has not been resolved (Hartshorne 1998).

1.5.3.2.1 Modulation of MLCP

As the phosphatase responsible for dephosphorylation of MLC_{20}, it is the principal target for mechanisms involving Ca^{2+} sensitisation, which is achieved though inhibition of MLCP. Inhibition of MLCP, occurs primarily through three mechanisms; firstly phosphorylation of Thr696 within the regulatory (MYPT1) subunit of MLCP has been shown to inhibit MLCP activity (Ichikawa et al. 1996; Kimura et al. 1996; Feng et al. 1999). This occurs primarily through rho-kinase (Kimura et al. 1996) activated as part of the G-protein signal transduction pathway, via Rho-guanine nucleotide exchange factors (Somlyo et al. 2000). Binding of GPCR also results in the second mechanism of Ca^{2+} sensitisation through MLCP. Activation of PLC results in the generation of DAG from the hydrolysis of PIP_2, DAG can activate PKC, which phosphorylates and activates CPI-17, a potent inhibitor of the catalytic subunit of MLCP (Eto et al. 1995; Senba et al. 1999). Arachidonic acid also plays an important role in the mechanisms of Ca^{2+} sensitisation, it mediates the dissociation of the holoenzyme and therefore inactivation of MLCP, resulting in a seven fold reduction in myosin-targeted phosphatase activity (Gong et al. 1992). In addition to this it is also able to activate rho-kinase (Fu et al. 1998; Araki et al. 2001) and atypical PKC (Gailly et al. 1997) therefore enhancing those Ca^{2+} sensitisation pathways.

1.5.3.3 Calmodulin

Calmodulin is a ubiquitously expressed protein, involved in a variety of Ca^{2+}-dependent cellular processes. It is 148 residues long (17 KDa), with a dumbbell shape, having two globular domains connected by a long central helix (Babu et al. 1985; Ikura et al. 1992). At low $[Ca^{2+}]_i$ calmodulin exists in the cytosol and does not interact with target proteins, upon stimulation of the cell and influx of Ca^{2+} through L-type VOCC or release from the SR, Ca^{2+} is able to bind and activate calmodulin. Each of the two globular domains has two Ca^{2+} binding sites, and upon binding Ca^{2+}, calmodulin undergoes a conformational change allowing it to modulate the activities and function of a number of enzymes and proteins, including; MLCK, caldesmon and PMCA as previously discussed (Word 1995).

1.6 Prostaglandins

Prostaglandins (PG) are a family of bioactive 20-carbon chain lipids derived from arachidonic acid, and are a subfamily of the eicosanoids. They are biological lipids, which act in an autocrine and paracrine fashion and are produced by all nucleated cells, except lymphocytes. There are five prostaglandins, involved in a wide range of biological functions; platelet aggregation, stomach acid secretion in addition to both contraction and relaxation of smooth muscle. There are five prostaglandins, named alphabetically in order of discovery; $PGTXA_2$, PGD_2, PGE_2, PGI_2 and $PGF_{2\alpha}$.

The two principal prostaglandins within the uterus are $PGF_{2\alpha}$ and PGE_2, where they play a major role in both the initiation and maintenance of labour and have also been suggested to be the cause of preterm labour.

1.6.1 Prostaglandin $F_{2\alpha}$

$PGF_{2\alpha}$ is one of the five prostaglandins, and is the principal prostaglandin in the uterus, where it functions as a uterotonic. As such expression is tightly regulated by synthesis and degradation, while function is dependent on $PGF_{2\alpha}$ expression, it is also dependent on the expression of its receptor (FP). Aberrant levels correlate with pre-term birth (Olson 2005) and endometriosis (Jabbour et al. 2004).

1.6.1.1 Function

$PGF_{2\alpha}$ is a uterotonic, increasing the contractility of the myometrium. It has been proposed that $PGF_{2\alpha}$ plays a role in parturition, there are several lines of evidence to suggest this; 1) $PGF_{2\alpha}$ increases contractility, 2) concentrations increase towards parturition, 3) labour can be induced by $PGF_{2\alpha}$ administration and 4) inhibition delays parturition.

$PGF_{2\alpha}$ is a myometrial stimulant. It is strong enough to initiate phasic contractility in quiescent tissue (Reiner et al. 1976), while it increases both the amplitude, duration and frequency of spontaneous contractility, in addition to an increase in basal tone (Reiner et al. 1976; Crankshaw et al. 1992; Phillippe et al. 1997; Sharif 2008). Paradoxically there has been one study in which very low $PGF_{2\alpha}$ concentration (10^{-12}) resulted in a reduction in basal tone,

without changing other force parameters (Khan et al. 2008), although no reasoning was given and no further studies have reproduced this effect.

While $PGF_{2\alpha}$ increases myometrial contractility, the uterus also display a change in sensitivity towards $PGF_{2\alpha}$; increasing towards parturition. The myometrium is most sensitive to $PGF_{2\alpha}$ on day 10, after which time it falls until day 18 where sensitivity starts to increase, continuing until parturition (Reiner et al. 1976; Crankshaw et al. 1992). Whilst the uterus is more sensitive to $PGF_{2\alpha}$ on day 10, the maximal tension is achieved at the end of gestation (Crankshaw et al. 1992). In addition to gestation affecting sensitivity and the degree of mechanical output, the anatomical area also affects the amount of force produced by $PGF_{2\alpha}$, with the ovarian portion of the myometrium of non-pregnant rats giving a greater mechanical output than the cervical section to the same concentration of $PGF_{2\alpha}$ (Oropeza et al. 2002). While longitudinal myometrial strips are more sensitive to $PGF_{2\alpha}$ than circular smooth muscle at all stages of pregnancy (Tuross et al. 1987).

$PGF_{2\alpha}$ stimulates myometrial contractility, it is also able to initiate parturition in both human and animal models. In a human study of oral $PGF_{2\alpha}$, $PGF_{2\alpha}$ was sufficient to induce labour in 16 out of 20 women (Karim et al. 1971) whilst a 15-hydroxyprostaglandin dehydrogenase hypomorphic mouse, which lack the enzyme responsible for degrading $PGF_{2\alpha}$, so increasing endogenous $PGF_{2\alpha}$, was able to overcome the progesterone block to initiate pre-term labour (Roizen et al. 2008). Investigations of $PGF_{2\alpha}$ ability to induce labour are limited due to the lack of clinical need, due to the existence of better drugs, such as oxytocin. As such $PGF_{2\alpha}$ is not used clinically to induce labour, rather it is utilised in abortions and for post-partum haemorrhage, were long strong contractions are required.

While $PGF_{2\alpha}$ may not be the best drug for the initiation of labour, it offers an attractive target as a tocolytic, as high $PGF_{2\alpha}$ concentrations correlate with infection induced pre-term labour (Mitchell et al. 1978; Sellers et al. 1981; Goldenberg et al. 2000). There are two ways in which to inhibit $PGF_{2\alpha}$; firstly by use of a specific $PGF_{2\alpha}$ receptor (FP) antagonists, or by a reduction in its synthesis. Currently there are no specific inhibitors of $PGF_{2\alpha}$ synthesis, although experimentally indomethacin, an inhibitor of PGHS-1 and -2 (see prostaglandin synthesis), is used to reduced the levels of all prostaglandins. Due to the lack of specific inhibitors for $PGF_{2\alpha}$ synthesis, only FP antagonists or transgenic animals lacking the FP receptor will be discussed, to ensure that response is due to $PGF_{2\alpha}$ and not other prostaglandins.

In *in vitro* studies, FP antagonists have been shown to reduce spontaneous contractility, while also reducing the amount of force $PGF_{2\alpha}$ is able to produce in pregnant rat myometrial tissue (Hirst et al. 2005; Doheny et al. 2007). In human pregnant and non-pregnant, spontaneous, oxytocin and phenylephrine-induced contractions showed a significant attenuation by the FP antagonist, presumably as a result of inhibition of action of basal $PGF_{2\alpha}$ (Friel et al. 2005). These results were repeatable in *in vivo* studies, were it was shown that FP inhibitors reduced spontaneous and $PGF_{2\alpha}$ induced contractility in a dose dependent and reversible manner, although in this study oxytocin-induced contractility only shown a decreasing trend and failed to reach significance (Chollet et al. 2007; Cirillo et al. 2007). In addition to a reduction in contractility profiles, *in vivo* studies also show a propensity for FP antagonists to delay labour, whether that be spontaneous labour, or pre-term labour induced by; $PGF_{2\alpha}$, RU 486 (progesterone antagonists), or lipopolysaccharide (LPS)(via inflammation). FP antagonists significantly delayed parturition, culminating in higher birth

weight and a higher proportion of live offspring when delivered naturally or by caesarean operation performed at expected term (Peri et al. 2002; Hirst et al. 2005; Chollet et al. 2007; Cirillo et al. 2007; Goupil et al. 2010).

A problem with antagonists, is the degree of specificity with which they act. In regard to FP antagonists the studies described above were carried out using three different FP antagonists, all reportedly FP-specific; PDC113.824, THG113.31 and AS604872, giving credence to the suggestions that the effects seen are due to the antagonism of FP rather than an off target effect. Similar effects were seen to FP antagonists in a study of mice lacking the FP receptor, giving similar results. There has been one study of mice lacking the FP receptor, similar to FP antagonists it was found that FP deficient mice were unable to deliver pups. Homozygote could become pregnant and gestation occurred normally, but parturition failed, although pups could be rescued by caesarean operation shortly before or at expected term (Sugimoto et al. 1997).

While experimentally, inhibition of $PGF_{2\alpha}$ both *in vitro* and *in vivo* have resulted in a decrease in myometrial contractility and the postponement of labour, to date there has yet to be a human clinical trial for an FP antagonists. Although there is currently no data for human clinical trials for FP antagonists, in the past inhibitors of $PGF_{2\alpha}$ synthesis have been trialled. Unfortunately both PGHS-1 and-2 inhibitors, both upstream synthases of PGs have detrimental effects to both mother and foetus (for reviews see London et al. 2000; Olsen 2005).

1.6.1.2 Expression

The functional effects of $PGF_{2\alpha}$ are dependent upon both the expression of $PGF_{2\alpha}$, and the expression of its receptor, FP. The expression of $PGF_{2\alpha}$ is difficult to investigate. It is synthesised and immediately secreted, can both diffuse through lipid bilayers and is transported across membranes, and is also rapidly degraded. Due to this, investigations into expression have relied upon changes in components of the synthesis and degradation pathways.

While myometrial levels of $PGF_{2\alpha}$ have not been identified, it is known that both amnion and uterine venous plasma $PGF_{2\alpha}$ increases in pregnancy, and through gestation (Molnar et al. 1990a). During birth $PGF_{2\alpha}$ metabolites rise with cervical dilation, continuing to increase through early labour into late labour (Mitchell et al. 1978; Sellers et al. 1981). In addition to this increase in $PGF_{2\alpha}$ leading to and through parturition, there is also a correlation between elevated amniotic $PGF_{2\alpha}$ concentrations and infection associated pre-term labour, responsible for around 40% of pre-term births (Mitchell et al. 1978; Sellers et al. 1981; Goldenberg et al. 2000).

1.6.1.2.1 Synthesis

Prostaglandins are not stored within the cell, but rather synthesised and immediately secreted when needed. All five family members are synthesised in a three step process, starting from the liberation of arachidonic acid from membranes, oxidation and reduction of arachidonic acid to the prostaglandin intermediate PGH_2 and finally the conversion from the intermediate PGH_2 by terminal synthases to the five prostaglandins (see Figure 1.6.1.2.1 for overview of $PGF_{2\alpha}$ synthesis).

Arachidonic acid, a 20-carbon carboxylic acid, polyunsaturated fatty acid present in phospholipids, found in the membranes of cells throughout the body, can be liberated by two mechanisms. In the first, phospholipase A_2 directly converts phospholipids to arachidonic acid, alternatively arachidonic acid can be produced indirectly by PLC. In gestation it is the first, phospholipase A_2, that is unregulated, allowing for the upregulation of the PGs (Zhang et al. 1996; Gibb 1998).

The second step in the synthesis of prostaglandins is a two-step process, of oxidation and reduction, converting arachidonic acid to the intermediate PGH_2. This step is mediated by two isoforms of prostaglandin endoperoxide H synthase, PGHS-1 and -2, also referred to as cyclo-oxygenase-1 and -2 (COX-1 and -2) (Olson 2003; Tsai et al. 2010). The first step is the dioxygenation of arachidonic acid, to give 9-11 endoperoxide and 15-peroxide group, this forming the unstable intermediate PGG_2 , the 15-peroxide group is then reduced to an alcohol forming PGH_2 (Smith et al. 2000). PGHS-1 and -2 have dual catalytic capabilities, allowing it to act as an endoperoxidase and cyclo-oxygenase activity, with crystallographic information showing two distinct binding sites, a peroxidise site and a cyclo-oxygenase site (Tsai et al. 2010). They are thought to act as homodimers on the luminal side of the nuclear envelope and endoplasmic reticulum membranes, as dissociation of the homodimer, inactivates both PSHS-1 and -2 (Xiao et al. 1998; Smith et al. 2000).

PGHS-1 and -2 are mediated by two distinct genes (Smith et al. 2002) but have high homology in their 576 and 587 amino acid sequence, of around 65%, with the residues thought to be important for catalysis conserved. Due to the high conservation of PGHS-1 and -2 they have similar affinities (Km) and capacities (Vmax), but are thought to have different physiological effects (Olson 2003).

Generally PGHS-1 is constituently expressed, and is referred to as the housekeeping enzyme, whereas PGHS-2 is inducible, by pro-inflammatory cytokines amongst others, with up to an 80-fold increase (Otto et al. 1995; Hertelendy et al. 2004). In uterine smooth muscle there is some debate over the expression and activity of PGHS-1 and -2. It is agreed that PGHS-1 levels do not change in pregnancy and labour, PGHS-2 on the other hand has been suggested to remain the same, decrease and increase, although more studies suggest that activity increases during labour (Moonen et al. 1984; Zuo et al. 1994; Dong et al. 1996; Moore et al. 1999; Erkinheimo et al. 2000; Tsuboi et al. 2000).

The third and final stage of $PGF_{2\alpha}$ production can be achieved in three distinct pathways. The principal pathway for $PGF_{2\alpha}$ production is through the reduction of the intermediary PGH_2 by 9, 11-endoperoxide reductase (prostaglandin F synthase (PGFS)), giving the principal 9α, 11α- $PGF_{2\alpha}$. An alternative pathway culminating in the production in 9α, 11α-$PGF_{2\alpha}$ is by reduction from PGE_2 by 9-ketoreductase. While the stereoisomer (9α, 11β-$PGF_{2\alpha}$) can be produced from PGD_2, by 11-ketoredutase (for reviews see Watanabe, 2000; Helliwell et al. 2004). While 9α, 11β- $PGF_{2\alpha}$, is not the principal $PGF_{2\alpha}$, it has been shown in uterine smooth muscle to result in contractility, similar to its isomer (Giles et al. 1991; Mitchell et al. 2005).

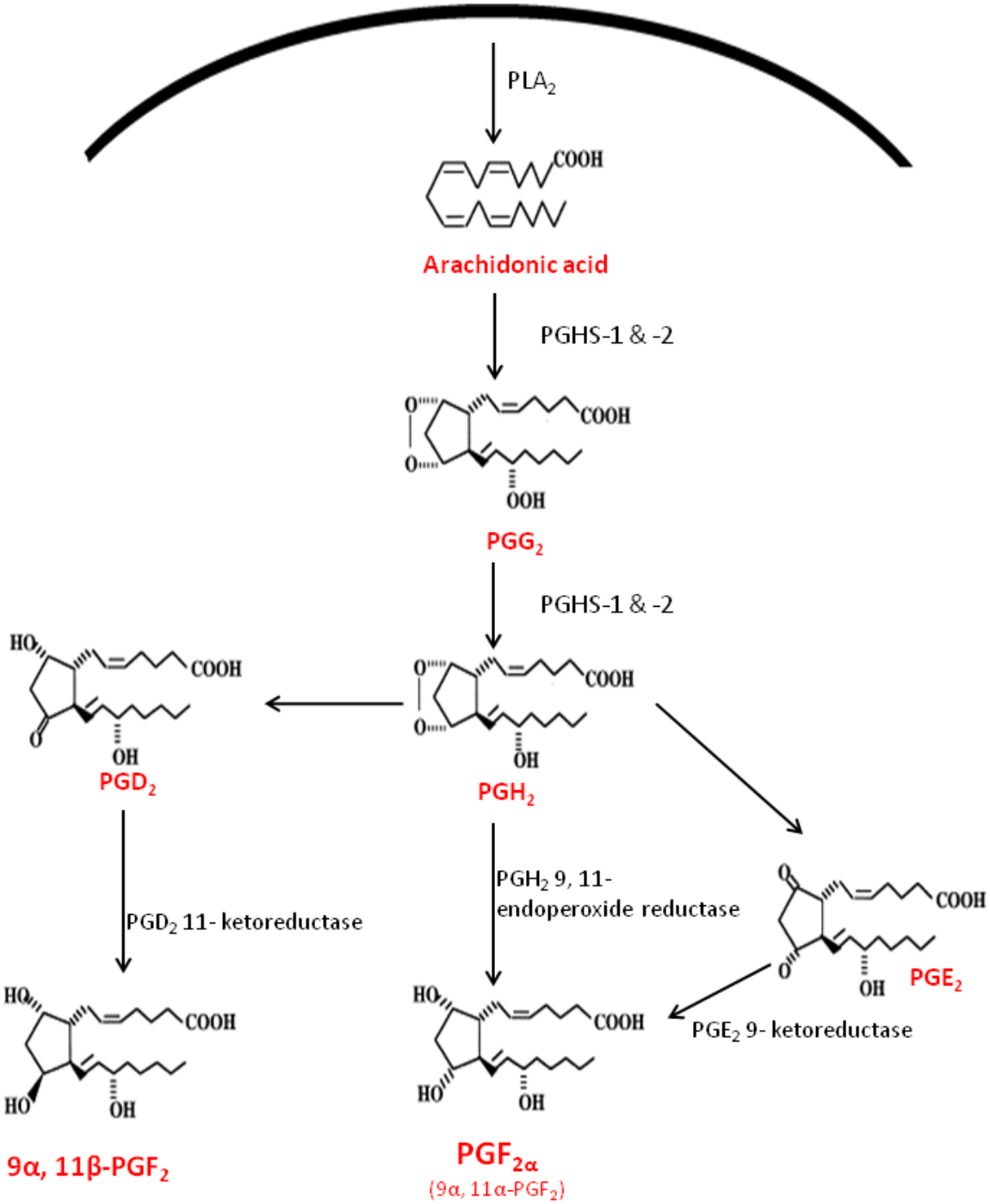

Figure 1.6.1.2.1 Schematic of $PGF_{2\alpha}$ synthesis pathway. Arachidonic acid is liberated from the cell membrane by phospholipase A_2, which is then converted to the intermediary PGH_2 by prostaglandin endoperoxide H synthase. $PGF_{2\alpha}$ is then produced in the classical pathway by PGH_2 9, 11- endoperoxide reductase, or from PGE_2, by PGE_2 9- ketoreductase . The functionally active stereoisomer can also be produced, by PGD_2 by PGD_2 11- ketoreductase. (Modified from Watanabe 2002)

1.6.1.2.2 Prostaglandin transport

Considering the importance of not only $PGF_{2\alpha}$, but all prostaglandins in physiological processes in both health and disease, little investigation has occurred in regard to the transport of prostaglandins. It is thought that prostaglandin synthesis starts in the ER, due to the localisation of PGHS homodimers on the luminal side of the ER. The product of which PGH_2, due to its non-polar nature, freely diffuses through the ER membranes, where it is converted to the more polar terminal prostanoids in the cytosol. From the cytosol they can diffuse freely across the membrane or be transported, this is also the mechanism needed for degradation, as little prostaglandin 15-dehydrogenases are found in either the blood or plasma (Schuster 1998; Schuster 2002).

Transport of anions across membranes by diffusion does occur, but at relatively low levels, often at a rate too low to maintain biological functions, which is thought to be the case for prostaglandins, highlighting the need for a transporter (Schuster 1998). The prostaglandin transporter (PGT) is a member of the 12-transmembrane solute carrier organic anion transporter2A1 family, (Kanai et al. 1995; Lu et al. 1996; Schuster 1998) which is able to mediate both influx and efflux of $PGF_{2\alpha}$ (Schuster 1998; Schuster 2002; Banu et al. 2003) and has been identified in uterine smooth muscle, increasing towards parturition (Banu et al. 2005). Unfortunately due to limited studies, PGT in the myometrium and their functional role in contractility is at this time unknown.

1.6.1.2.3 Prostaglandin degradation

While synthesis of $PGF_{2\alpha}$ is intimately linked to its effects, the rate of degradation is also critical to its biological concentrations, and therefore effects. The first step in $PGF_{2\alpha}$ degradation is the oxidation of the 15-hydroxyl group by the NAD^+-dependent 15-hydroxyprostaglandin dehydrogenase (PGDH), giving the biologically inactive 15-keto-13,14-dihydro $PGF_{2\alpha}$ (Okita et al. 1996)

There is no or little PGDH activity in the blood or plasma of a number of species, but activity is high inside cells. Evidence of this culminated in the suggestion of the two step model of metabolic clearance (Schuster 1998; Schuster 2002); suggesting that firstly prostaglandins are transported into the cell where they can then be metabolised by PGDH. This has been re-created in a number of cellular models, reproducing metabolism of exogenous $PGF_{2\alpha}$ (Nomura et al. 2004).

The levels of PGDH are functionally vital in the myometrium, in maintaining uterine quiescent during gestation and initiating parturition. During pregnancy PGDH activity is high, which is thought to be due to progesterone, a positive modulator (Greenland et al. 2000). While PGDH activity is high in pregnancy, it does not change through gestation, although it does drastically decrease in both term and pre-term labour (Giannoulias et al. 2002). The importance of a reduction in PGDH in parturition is made clearer when studied in relation to pre-term birth. In a study of idiopathic pre-term labour not associated with infection, there was a marked reduction in PGDH expression activity within the chorion (Sangha et al. 1994). It is the chorion that acts as a barrier between the amnion and the myometrium, and so a reduction in chorion PGDH increases myometrial $PGF_{2\alpha}$ (Challis et al. 1999). In addition to there being a reduction in idiopathic preterm labour, during infection chorion trophoblasts

are destroyed which therefore dramatically reduce PGDH activity, and so PGDH also plays a role in infection-induced pre-term birth (Van Meir et al. 1996; van Meir et al. 1997).

1.6.1.3 $PGF_{2\alpha}$ receptor (FP)

It was originally thought that the prostaglandins directly interact with the cell membranes, but due to their differing profiles it was suggested that they interact with receptors and that each prostaglandin has its own receptor (Kennedy et al. 1982). With the use of specific antagonists these receptors were further characterised, giving the classification that still stands today (Coleman et al. 1994). DP receptor for PGD_2, IP receptor for PGI_2, TP receptor for $PGTXA_2$, FP receptor for $PGF_{2\alpha}$ and four EP receptors ($EP_{1\text{-}4}$) for PGE_2 . It was determined that while each prostanoid binds preferentially to its own receptor, they are able to interact with other PG receptors (Coleman et al. 1994).

The FP receptor has been cloned from a number of species (Abramovitz et al. 1994; Sugimoto et al. 1994; Sakamoto et al. 1995) and has been determined to be a G-protein coupled receptor, with seven transmembrane domains, an extracellular N-terminal and intracellular –COOH, typical of the rhodopsin-type G-protein coupled receptors (Woodward et al. 2011). It has a predicted mass of 40 KDa (Sakamoto et al. 1995), and ligand binding studies, suggest that $PGF_{2\alpha}$ displays a high affinity (<10nM) binding for its receptor, with a Kd of 1.32nM reported from recombinant FP receptors in COS cells (Coleman et al. 1994).

The FP receptor has been shown to be a G-protein coupled receptor, functional studies have determined that FP is predominantly Gq-coupled, and so through activation of PLC, increases DAG and IP_3 (Woodward et al. 2011). While this is the predominant pathway,

were most of the evidence from uterine smooth muscle points, there has been studies from other tissues, suggesting that FP is also coupled G_{12}/G_{13}, activating Rho (Pierce et al. 1999) and G_i, MAP kinase pathway (Melien et al. 1998), although this has not been identified in uterine smooth muscle.

Similar to PGHS, expression of the FP receptor change with pregnancy and labour. In human pregnancy expression decreases by 45% compared to levels found in non-pregnant myometrium, while levels were higher in labouring samples (Matsumoto et al. 1997; Brodt-Eppley et al. 1999). There is also a correlation between high FP receptor levels in pre-term birth, with higher levels found in pre-term birth with and without labour compared to term-pregnancy without labour (Brodt-Eppley et al. 1999). Similar to human tissue, rat myometrial FP receptor expression increases significantly from late gestation until delivery, returning to pre-partum levels by one day post-partum (Brodt-Eppley et al. 1998; Al-Matubsi et al. 2001). This leads to the suggestion that FP receptors play a pivotal role in the maintenance of quiescence during gestation and the initiation of parturition. While FP receptors obviously play a vital role, the mechanism of regulation in the myometrium is currently unknown.

1.6.1.4 Proposed mechanism of action

The importance of $PGF_{2\alpha}$ is in no doubt, there have been numerous studies regarding its role in both normal physiology and pathological conditions. As such it is surprising what little information is known regarding its mechanism of action, especially in the myometrium.

Due to the importance of $[Ca^{2+}]_i$ in the contractility of the uterus, it is hardly surprising that both intracellular and extracellular Ca^{2+} sources have been shown to be important to the full effects of $PGF_{2\alpha}$ (Parkington et al. 1999; Coleman et al. 2000; Ruttner et al. 2002). Although the relative contributions are still debated; some studies suggest only extracellular Ca^{2+} is needed (Molnar et al. 1990b; Perusquia et al. 1992), while others show release from the store that is adequate to cause contraction (Reiner et al. 1976; Villar et al. 1986). This release from the store is thought to be mediated through an increase in IP_3, through the pertussis insensitive Gq (Maka et al. 1993; Phaneuf et al. 1993; Phillippe et al. 1997) known to associate with the FP receptor (Coleman et al. 1994) although again there is some controversy that the amount of IP_3 produced by $PGF_{2\alpha}$ is enough to result in adequate release from the store (Molnar et al. 1990b; Molnar et al. 1995).

Besides the importance of both intracellular and extracellular Ca^{2+}, of which there is debate regarding relative contributions, Ca^{2+} sensitisation has also been suggested to be the cause of $PGF_{2\alpha}$ increase mechanical output in uterine smooth muscle, although again these studies are limited, and do not look at the mechanisms of Ca^{2+} sensitisation (Izumi et al. 1996; Woodcock et al. 2006). Another way in which $PGF_{2\alpha}$ exerts its affects is through depolarisation, $PGF_{2\alpha}$ causes depolarisation, consisting of a slow wave of depolarisation, dependent on Na^+, followed by a fast train of action potentials, which is dependent on Ca^{2+} (Reiner et al. 1976). Again the mechanism of this depolarisation is as yet unresolved.

Limited studies have also suggested the involvement of ROC and influx of Ca^{2+} (Coleman et al. 2000). $PGF_{2\alpha}$ has also been suggested to result in a reduction in cAMP, although this is highly controversial, based on two studies; the first reporting a reduction in IBMX induced

cAMP by $PGF_{2\alpha}$ (Goureau et al. 1990) whilst the second reported that cAMP was not attenuated by $PGF_{2\alpha}$ (Molnar et al. 1987).

While the mechanisms of $PGF_{2\alpha}$ have not been studied in detail, it is probable that like many agonists, $PGF_{2\alpha}$ works through multiple mechanisms synergistically to increase myometrial mechanical output. To date the mechanisms involved in $PGF_{2\alpha}$ response and their relative contributions are unknown.

1.7 Summary

In summary, the uterus is a spontaneously active muscle under myogenic control, modulated by hormonal stimuli. One of the critical hormones involved in parturition is $PGF_{2\alpha}$. Without $PGF_{2\alpha}$ parturition does not occur, while high levels correlate with pre-term birth, especially those caused by infection. While the functional role of $PGF_{2\alpha}$ as a uterotonic is understood, the mechanism by which it is able to increase mechanical output of myometrial tissue is unresolved.

1.8 Aims

The broad stroke of my study was to examine the force Ca^{2+} relationship in response to $PGF_{2\alpha}$ in late gestation pregnant rat uterine smooth muscle.

Specific aims of this study were:

- To determine the relationship between force and Ca^{2+} in pregnant rat myometrium in control conditions.
- To study the force Ca^{2+} relationship induced by $PGF_{2\alpha}$ compared to those in control conditions.
- To study the mechanism of $PGF_{2\alpha}$, specifically the role of Ca^{2+} release from the store, and Ca^{2+} entry pathways.

Chapter 2

Materials and methods

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2.1 Tissue preparation

Wistar rats were chosen as our model because they have been regularly used in our laboratory and have little variation in gestation. Virgin females were mated on day 0 (PM), and humanely killed by cervical dislocation under CO_2 anaesthesia on day 22 (AM), full term, in accordance with UK Home Office legislation. Once neonates were removed, uterine horns were dissected and carefully cleaned. Uterine tissue was kept in physiological saline solution (PSS) for the remainder of time, unless otherwise stated.

2.1.1 Longitudinal myometrial strips

Uterine tissue was pinned to the bottom of an agar-filled petri dish and with the use of forceps and scissors, small strips of the appropriate length, of longitudinal muscle layer were removed from the underlying circular smooth muscle layer and endometrium. Longitudinal muscle strips were loaded with the appropriate Ca^{2+} sensitive indicators, Indo-1 AM or Fluo-4 AM, for measuring $[Ca^{2+}]_i$ using photometric or confocal imaging systems, respectively, combined with simultaneous force recordings.

2.1.2 Cell isolation

Longitudinal myometrial strips from the freshly obtained uterus were cut into small pieces and incubated in 2ml modified Hanks balanced salt solution (HBSS) (see composition below); for 30 min at 36°C with gentle agitation and changing the solution twice.

Enzyme digestion was achieved with Liberase Blendzyme 3 (0.28 Wunsch/ml); 5ml modified HBSS; for 50-60 minutes, at 36°C with gentle agitation. After enzyme digestion strips were removed and washed thoroughly in modified Kraft-Brühe (KB) media (for composition of KB media see below). Tissue was then incubated for 30mins in 1.5ml KB media at room temperature. Trituration was begun with a fire polished glass Pasteur pipette with 2mm diameter tip, the suspension obtained was filtered on a 20µm nylon mesh. Freshly isolated myocytes were kept in KB media until loaded with Fluo-4, not exceeding 4 hours.

2.2 Measuring isometric force

To investigate contractility, longitudinal tissue strips of approximately 1 x 2 mm were clamped by two aluminium foil clips at both ends, transferred to a small organ bath (500 µl) and fixed to the bottom of experimental chamber at one end and with a Fort10g isometric force transducer (WPI, Hertfordshire, UK) at the other (Figure 2.2a). PSS or appropriate solutions were perfused at 3.5 ml/min through a magnetic valve controlled gravity fed system of syringes and heated to 36.5°C throughout (Figure 2.2b).

Passive tension was set by use of a reference high-K^+ solution (40 mM KCl). Passive tension was set at 50 % of high-K^+ peak mechanical response, tissue was left until spontaneous contractions became regular and stable. If the tissue failed to spontaneously contract after 60 minutes, but was responsive to high-K^+, it was classified as quiescent.

In all protocols an initial 40 second application of high-K^+, the initial amplitude of which was used to normalise both force and Ca^{2+} data, taken for 100%. Tissue was left to stabilise for at least 10 minutes after high-K^+ induced response before start of experiments.

Electrical signals from the force transducer were amplified and recorded by axon CNS datadigi 1440A data digiboard (Scientifica, Sussex, UK), using Axon software (Axoscope version 10.0, Molecular devices, Berkshire, UK), at a sampling rate of 1 kHz (Figure 2.2b), and measured in mN.

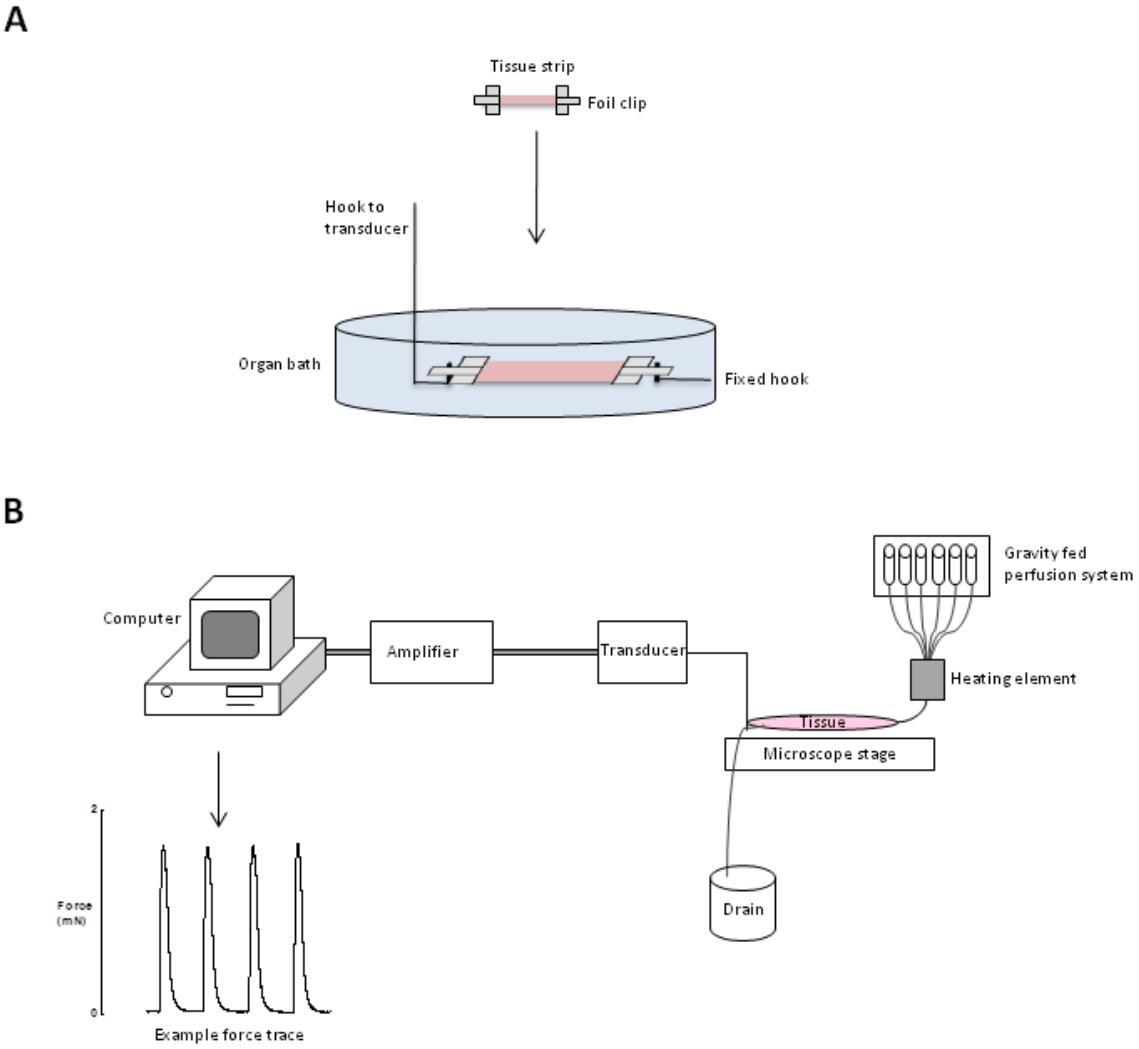

Figure 2.2 Diagram of the system used to measure isometric force. (A) Longitudinal myometrial strip (1x2mm) attached to two aluminium clips. Clips are attached to a fixed hook in the 500µl organ bath and a free hook attached to an isometric force transducer. The bottom of the bath is made of a glasscoverslip enabling bath to be place over microscope objectives for simultaneous force and $[Ca^{2+}]_i$ recordings. (B)Organ bath placed on top of microscope stage and continuously perfused by gravity fed system at 3.5ml/min and heated to 36.5°C. Isometric force transducer is routed through an amplifier and recorded on a computer by axon software at a sampling rate of 1KHz.

2.3 Measuring $[Ca^{2+}]_i$

2.3.1 Photometric system, Indo-1

Indo-1 AM was chosen as the Ca^{2+} indictor for simultaneous force $[Ca^{2+}]_i$ measurements on the photometric system. Optimisation of indo-1 AM concentration was determined to be 12.2µM, loaded for 3 hours 30 minutes at room temperature. In these conditions complete hydrolysation of the AM ester occurred, without excessive formaldehyde accumulation. The use of indo-1 AM did not compromise tissue function, gave a good signal, which lasted for the duration of the experiment (2 hours).

Indo-1 AM (50µg) was prepared in a solution of 50 µl DMSO containing 12.5mg pluronicF-127, added to 4ml PSS. 5 myometrial strips were incubated into 2ml of the indo-1 PSS for 3 hours 30 minutes at room temperature, with gentle agitation.

Ca^{2+} measurements were obtained for the ratio of emission wavelengths of 400nm and 500nm, while excited at 355nm. Excitation of 355nm was provided by xenon lamp and monochromator graticule (Cairne, Kent, UK), and focused on the sample with x10 (N.A. 0.30) objective (Olympus, Essex, UK), whilst the two emissions wavelengths were detected by photomultiplier tubes (Cairne, Kent, UK), and amplified and converted to a digital signal by axon CNS datadigi 1440A data digiboard (Scientifica, Sussex, UK). Signals were recorded at a sampling rate of 1 KHz, using Axoscope software (version 10.0, Molecular devices, Berkshire, UK) (Figure 2.3.1.1). A typical example of changes in fluorescence measured at 400nm and 500nm and their ratio is shown in Figure 2.3.1.2.

Due to the difficulties in calibrating $[Ca^{2+}]_i$ for each individual experiment, all Ca^{2+} analysis is expressed as a percentage of initial high-K^+ response, unless otherwise stated. Additionally it was not confirmed that the fluorescent indicator was not saturated during experiments, this could have been achieved with the addition of high concentration of Ca^{2+} to the bathing solution at the end of experiments. While saturation was not tested, this would not have been an issue in the majority of experiments, as analysis was completed at lower than maximum $[Ca^{2+}]_i$ within the experiment. For experiments analysed at the top of Ca^{2+} trace, deep spikes remained, suggesting that the signal was not saturated.

For simultaneous measurement of force and $[Ca^{2+}]_i$ the experimental apparatus for force measurement previously described can be place on the stage of an inverted microscope, with the use of a glasscoverslip as the bottom of the chamber, both excitation and emission wavelengths can pass through, allowing for the simultaneous measurement of both force and $[Ca^{2+}]_i$. Data from Ca^{2+} recording and force is routed to the same data digiboard and recorded simultaneously using Axoscope software (Figure 2.3.1.3)

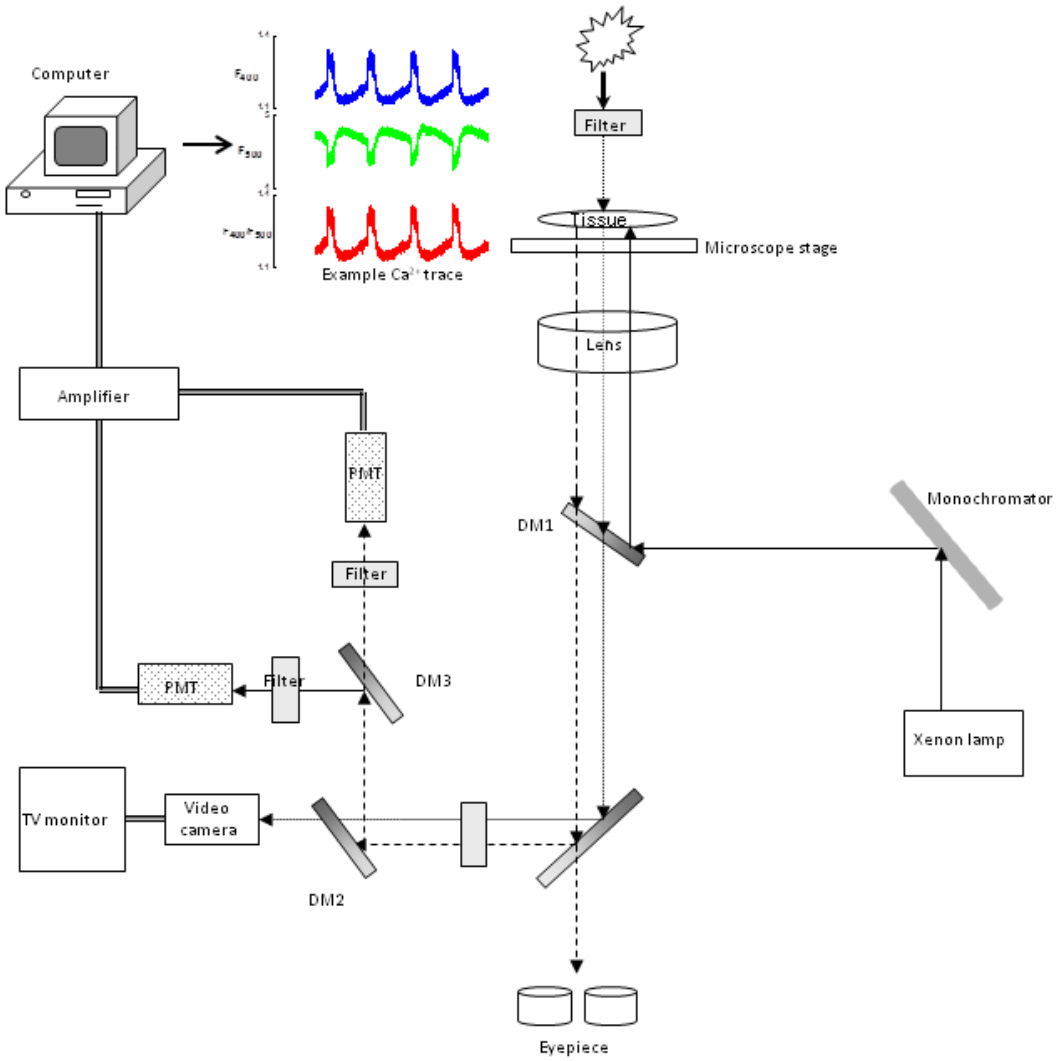

Figure 2.3.1.1 Diagram of system used to measure $[Ca^{2+}]_i$. Xenon lamp and monchromator graticule used to provide excitation of 355nm. The light is reflected towards the sample by the first dichroic mirror (DM1) and focused by use of a x10 objective (N.A. 0.30). The light passes back through DM1 and reflected through a sliding mirror through a diaphragm onto another dichroic mirror (DM2), where light either passes through to a video camera, so that the sample can be viewed on a TV monitor and towards the third and final dichroic mirror. DM3 splits the light and with emission filters, light of 400nm and 500nm passes to the photomultiplier tubes (PMT), routed through an amplifier before being recorded onto a computer by axoscope software at a sampling rate of 1kHz.

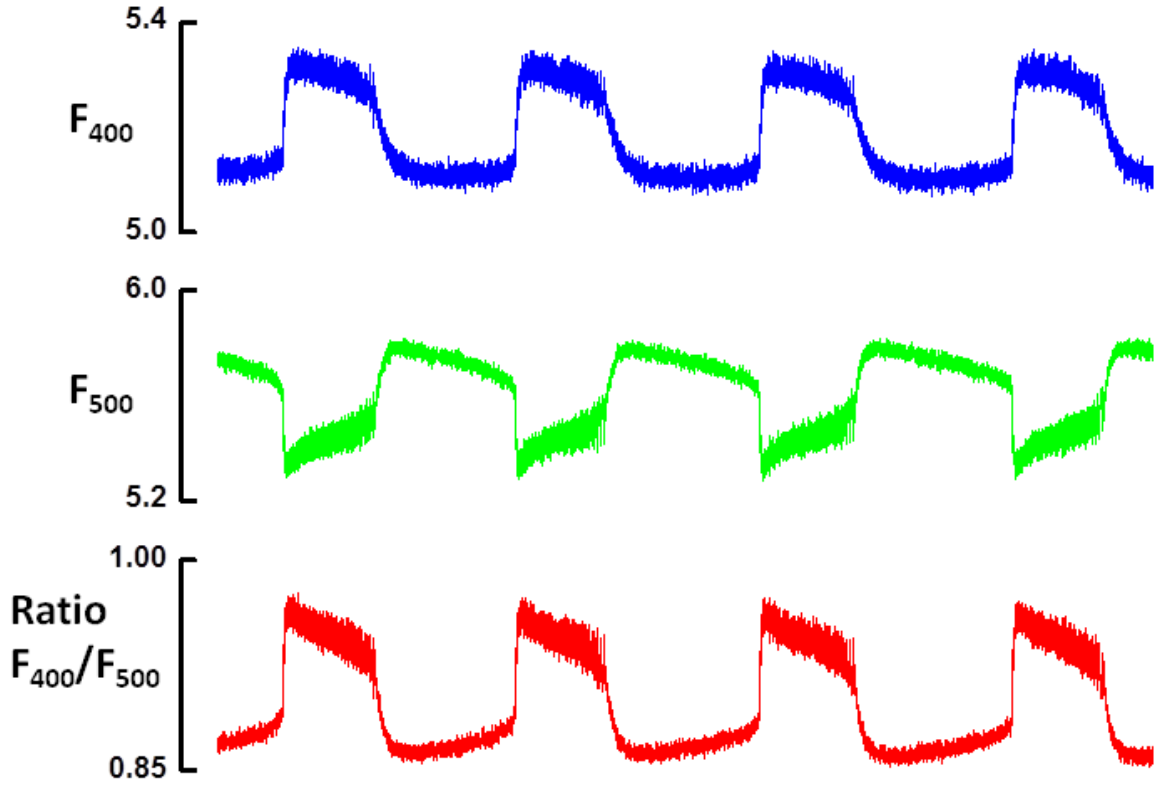

Figure 2.3.1.2 Example recording of how $[Ca^{2+}]_i$ change is determined for Indo-1 AM. Emission wavelength 400nm (blue) and 500nm (green) were recorded, before the ratio (red) was taken, giving the change in intracellular $[Ca^{2+}]_i$.

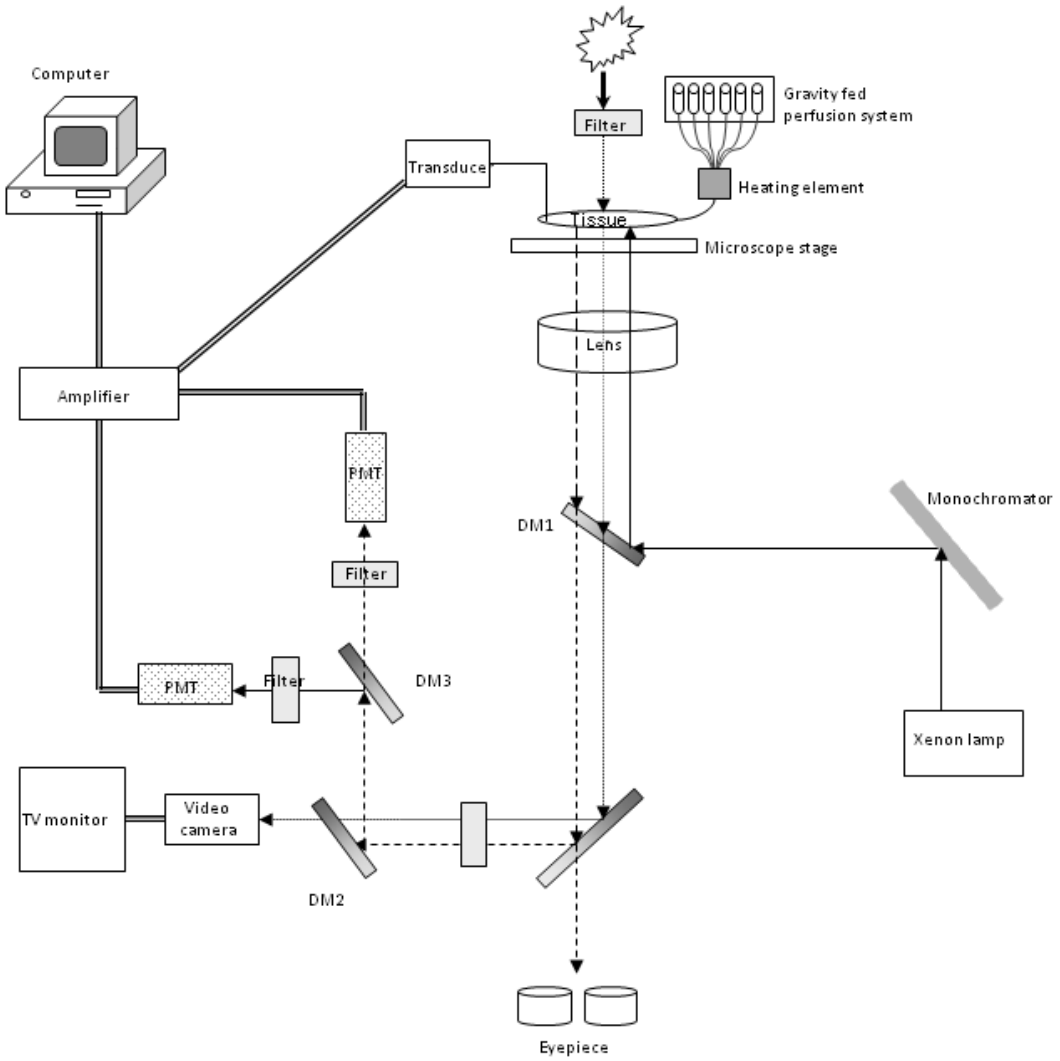

Figure 2.3.1.3 Diagram of system used to measure simultaneous $[Ca^{2+}]_i$ and force. In order to measure simultaneous force and $[Ca^{2+}]_i$, one end of the sample was attached to an isometric force transducer, this was routed to the amplifier/data digiboard from the photometric system.

2.3.2 Confocal microscopy, Fluo-4

2.4.2.1 Myometrial strips

Fluo-4 AM was used as the Ca^{2+} sensitive fluorescent indicator for all confocal microscopy work. Optimisation of Fluo-4 AM concentration for myometrial strips was determined to be 22.2µM, loaded for 3 hours. In these conditions complete hydrolysation of the AM ester occurred, without excessive formaldehyde accumulation. The use of Fluo-4 AM did not compromise tissue function, gave a good signal, which lasted for 2-3 hours.

Fluo-4 AM (50µg) was prepared in a solution of 50 µl DMSO containing 12.5mg pluronicF-127, added to 2ml PSS. 5 myometrial strips were incubated in 1 ml of the Fluo-4 PSS for 3 hours at room temperature, with gentle agitation.

Ca^{2+} measurements were obtained by use of a Nipkow discbased, confocal imaging system (PerkinElmer, MA, USA), connected to a sensitive iXon cooled charge-coupled device camera (Andor technology, Belfast, UK). Excitation was provided by an argon 488/568nm laser (PerkinElmer, MA, USA), at 488nM and focused by x20 objective (N.A. 0.72). Images were collected at 30 frames per second using Andor iQ software (Andor technology, Belfast, UK). (Figure 2.3.2)

For simultaneous measurement of $[Ca^{2+}]_i$ and force, one end of the strip was attached to a Fort10g isometric force transducer, the other to a fixed hook. Mechanical signal was recorded by another computer as previously described.

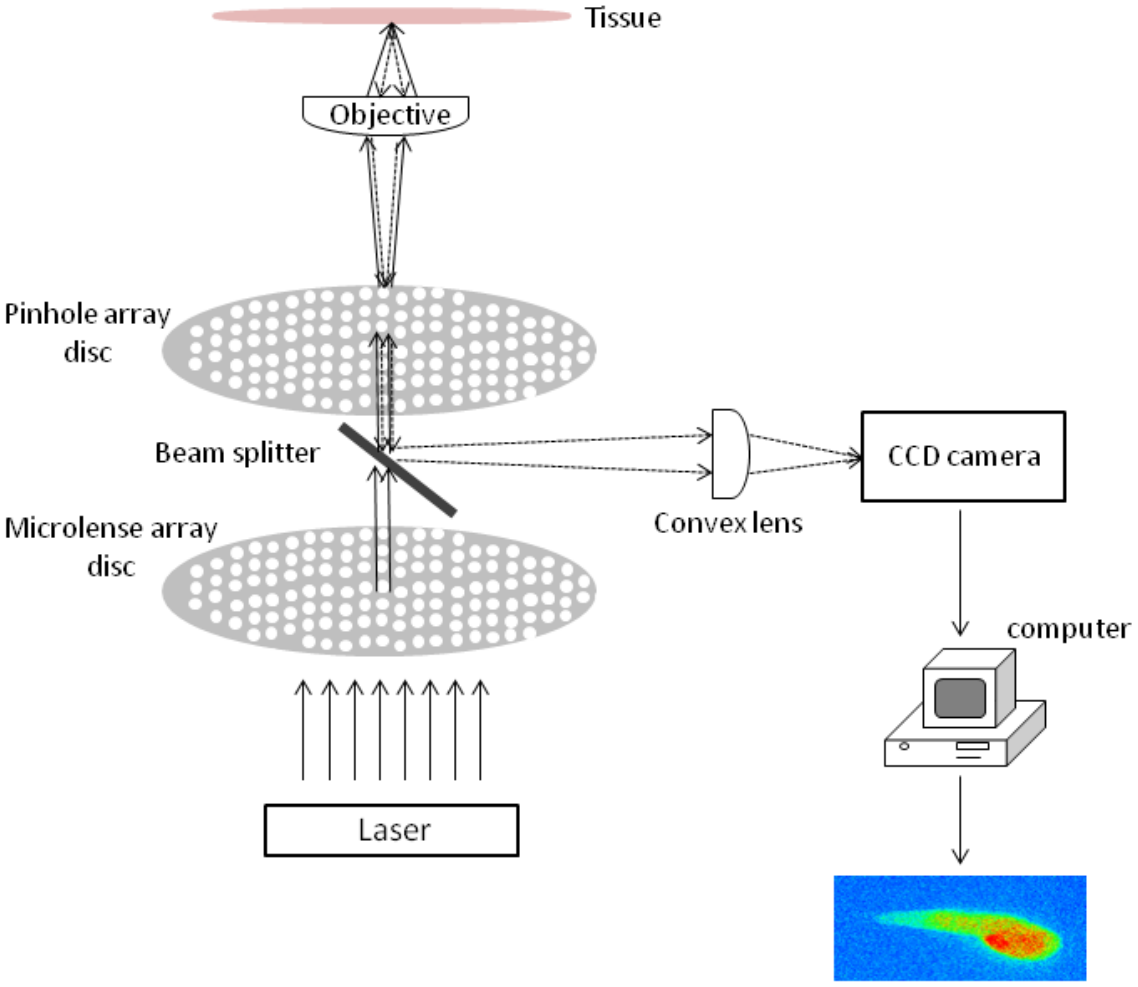

Figure 2.3.2 Schematic diagram of setup of confocal microscope to measure $[Ca^{2+}]_i$.
An argon 488/568mn laser provided excitation at 488nm, excess light is removed and excitation illumination further focused onto pinhole array disc by the microlense array disc. Light passes through the pinhole array, eliminating excess light and is focused onto sample by a x20 objective (N.A. 0.72) or x60 water objective (N.A. 1.25). Light passes back through the pinhole array disc and is reflected by the beam splitter through a convex lens to focus light onto the iXon cooled CCD camera, recorded onto a computer at 30 frames per second.

2.4.2.2 Isolated cells

Optimal concentration of Fluo-4 AM for isolated cells was determined to be 2.5µM, loaded for 20 minutes at room temperature. In these conditions complete hydrolysation of the AM ester occurred, without excessive formaldehyde accumulation. The use of Fluo-4 AM did not compromise cellular function, gave a good signal, which lasted the duration of the experiment.

1 mM stock Fluo-4 was prepared by addition of 46µl DMSO to 50µg aliquot Fluo-4 AM. Indicator was prepared by addition of 5µl DMSO containing pluronicF-127 acid (50mg pluronic acid plus 200 µl DMSO) to 5µl 1mM Fluo-4 to 2ml cell suspension, giving a final Fluo-4 AM concentration of 2.5µM. Cell suspension was then incubated at room temperature on the stage of a confocal microscope for 20 minutes, allowing cells to adhere to the glasscoverslip, after which time cells were slowly perfused with PSS solution.

Measurement of $[Ca^{2+}]_i$ was the same as used for myometrial strips. Again Confocal images were collected at 30 frames per second using x60 water objective (N.A. 1.25) (Olympus, Essex, UK).

2.5 Analysis and statistics

The software used to analyse data was OriginPro 8.6 (OriginLab corporation, Northampton, MA, USA). Statistics were performed in SPSS statistics 19 (IBM, Hampshire, UK). Distribution of data was determined by use of Shapiro-Wilk test and analysed using either paired-samples t-test or independent-samples t-test or the non-parametric versions; Wilcoxon signed rank test or Mann-Whitney U test. Statistical significance was taken as $p < 0.05$, with individual p values given in parentheses. The data is given as mean ± S.E.M or median ± I.Q.R. as appropriate, with '*n*' representing the number of experiments on different animals, unless otherwise stated.

2.6 Solutions and Chemicals

All chemicals from Sigma Aldrich (Gillingham, UK) and pH to 7.40 with 4M NaOH and 1M HCl as needed, unless otherwise stated.

Physiological saline solution (krebs): Freshly made, to the following constituents:

NaCl, 154 mM

KCl, 5.6mM

$MgSO_4$, 1.2mM

HEPES, 10.9mM

Glucose, 11.7mM

$CaCl_2$, 2mM

High potassium solution (40mM KCl): Freshly made, to PSS recipe with isosmotic substitution of NaCl for KCl.

NaCl, 119.6mM

KCl, 40mM

Modified Kraft-Brühe (KB) media: Freshly made, to the following:

KCl, 40mM

K_2HPO_4, 10mM

Taurine, 10mM

TES, 10mM

Sucrose, 11mM

Pyruvate, 5mM

Creatine, 5µM

EGTA, 50µM

K-glutamate, 100nM

BSA/ albumin, 1mg/ml

Stock immediately frozen -20˚C, used within 2 weeks.

Zero Ca^{2+} physiological saline solution ($0Ca^{2+}$): Substitution of $CaCl_2$ with 2mM of the Ca^{2+} chealator ethyl glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetra acetic acid (EGTA).

Modified physiological saline solution: Substitution of $MgSO_4$ for $MgCl_2$ (1.2mM), needed for Gd^{3+} and La^{3+} experiments, as $MgSo_4$ will precipitate out.

Modified zero Ca^{2+} physiological saline solution: Substitution of $MgSO_4$ for $MgCl_2$ (1.2mM), needed for Gd^{3+} and La^{3+} experiments, as $MgSO_4$ will precipitate out.

Modified Hanks balanced salt solution (HBSS): Made daily Ca^{2+}-free and Mg^{2+}-free HBSS (Life technologies, Paisley, UK) 40µM Ca^{2+} 1mg/ml albumin BSA.

Adenosine triphosphate (ATP): Reconstituted in dH_20 for a stock concentration of 10mM daily, working concentration 100µM prepared in PSS/0Ca PSS.

Carbachol (CCh): Reconstituted in dH_20 for a stock concentration of 100mM stored at +4°C, for maximum 4 months (efficacy previously confirmed in the laboratory). Working concentration of 100µM prepared in PSS/0Ca PSS.

Cyclopiazonic acid (CPA): Reconstituted in DMSO for stock concentration of 20mM stored at +4°C, working concentration of 20µM prepared daily in PSS/0Ca PSS/nifedipine.

Gadolinium (Gd^{3+}): Fresh 1mM stock concentration of $GdCl_3$ dH_20 prepared freshly when required, diluted to working concentration of 10µM in modified PSS/modified 0Ca PSS.

Lanthanum (La^{3+}): Fresh 10mM stock concentration of $LaCl_3$ dH_20 prepared daily when required, diluted to a working concentration of 10µM in modified PSS/modified 0Ca PSS.

Liberase Blendzyme 3: Reconstituted to manufactures instruction in HBSS, to 14 Wünsch/ml. Working concentration 0.28 Wünsch/ml in modified HBSS. (Roche Applied Science, Mannhein, Germany)

Nifedipine: Reconstituted in ethanol to a stock concentration of 10mM, stored at +4°C in dark. Working concentration 10µM prepared in appropriate solution.

Prostaglandin $F_{2\alpha}$: Stock concentration 1mM reconstituted in dH_20, aliquots stored at -20°C. Thawed aliquots stored at 4°C for a maximum of 5 days, diluted in appropriate solution for working concentrations of between 10nM - 5µM.

18-β-glycyrrhetinic acid (18-β-GA): Reconstituted in DMSO for a stock concentration of 50µM stored at +4°C, working concentration 50nM prepared in PSS.

Chapter 3

Ca^{2+} and contractility profiles in late gestation pregnant rat myometrium

Chapter 3

Ca^{2+} and contractility profiles in late gestation pregnant rat myometrium

3.1 Introduction

Ca^{2+} plays a pivotal role in the production of force in uterine smooth muscle. An increase in $[Ca^{2+}]_i$ occurs either through Ca^{2+} influx via VOCC or release from the SR. Ca^{2+}-calmodulin activates MLCK, leading to the phosphorylation of myosin light chains (MLC_{20}), resulting in activation of cross-bridge cycling and the production of force. Relaxation occurs upon dephosphorylation of MLC_{20}-P cross bridge cycling can no longer occur and force therefore falls.

In spontaneously active rat longitudinal myometrium, the increase in $[Ca^{2+}]_i$ results primarily from influx through VOCC (L-type Ca^{2+} channels) during the action potential (Shmigol et al. 1998b). Spike like action potentials (AP) are generated by an influx of Ca^{2+} through VOCC in addition to an increase in permeability to Na^+ (Shmigol et al. 1998b) peaking within approximately 10ms, after which repolarisation occurs, due to, voltage-, time- and Ca^{2+}-dependent inactivation of VOCC along with activation of voltage- and Ca^{2+}-dependent K^+ channels (Mironneau et al. 1980; Mironneau et al. 1981).

The mechanism of initiation of action potentials in the myometrium has not been elucidated, and as yet pacemaker cells have not been identified. It is thought to involve the rhythmic alteration in basal membrane potential, termed slow waves, that are seen in smooth muscle cells of myometrial tissue, although again the underlying ionic mechanism

responsible for this is yet to be fully resolved (Casteels et al. 1965; Sanborn 1995). Once membrane potential of the slow wave reaches threshold potential, a train of fast spike-like action potentials are generated on top of the slow wave (Kuriyama et al. 1976). It is this train of action potentials that generate Ca^{2+} spikes and force (Burdyga et al. 2009).

The duration of the train of action potentials and the frequency within each burst controls intracellular Ca^{2+} and thus is responsible for the control of myometrial contractility. Within a train of action potentials, a single action potential is associated with single Ca^{2+} spike and brief phasic contraction (Burdyga et al. 2009). During the burst of action potentials which result in a burst of Ca^{2+} spikes the phasic contractions due to slower kinetics of relaxation summate and produce complex titanic like phasic contractions (Mironneau 1973; Shmigol et al. 1998b). Depending on the frequency of action potentials (Ca^{2+} spikes) this results in either incomplete tetanus, where notches in force can easily be seen within the complex phasic contraction, or at higher frequencies of action potential mediate smooth titanic-like phasic contractions. Thus, the force produced is as a result of summation of individual phasic contractions associated with each action potential mediated Ca^{2+} spike (Mironneau 1973; Burdyga et al. 2009).

While the total contraction is dependent on the duration and frequency of trains of action potentials within the burst, it is also dependent on the ability of the tissue to propagate the action potentials between the smooth muscle cells via gap junctions and synchronise contraction of multiple muscle bundles. In the myometrium as in other phasic smooth muscles gap junctions provide the electrical communication between uterine smooth muscle cells (Cole et al. 1985). During gestation and in the non-pregnant uterus the density of gap junctions is low which is thought to ensure the limitation in spatial spread of action

potentials, which results in poor electrical coupling between smooth muscle cells and asynchronous activity leading to the maintenance of quiescence throughout gestation (Lammers et al. 1994). Towards the end of gestation and parturition, propagation of action potentials and velocity of conduction increases, due to an increase in the density of gap junctions, improving the synchronous activity needed to maximise recruitment of the muscle bundles to expel the foetus (Cole et al. 1985; Lammers et al. 1994; Doring et al. 2006).

The aim of this chapter was to characterise the force / Ca^{2+} relationship in longitudinal myometrial strips isolated from late gestation rat myometrium by simultaneous recording of $[Ca^{2+}]_i$ and force on small (1 x 2 mm) longitudinal myometrial strips loaded with the Ca^{2+} - sensitive indicator Indo-1.

3.2 Material and methods

Tissue

Longitudinal myometrial strips (1x 2 mm) from the ovarian portion of 22 day gestation Wistar rats were used throughout, obtained as described in Chapter 2.

Simultaneous measurements of force and $[Ca^{2+}]_i$

Simultaneous measurements of force and $[Ca^{2+}]_i$ was achieved, using photometric system as previously described (see Chapter 2 for details). Tissue strips were continuously perfused with PSS until stable spontaneous activity was observed or tissue classified as quiescent, if spontaneous activity did not start in 60 min. After a period of stable contractility and high-K^+ responses, PSS containing nifedipine (10µM) or 18-β-glycyrrhetinic acid (18-βGA) (50nM) was applied, to investigate the role of L-type VOCC in the control of Ca^{2+} signalling and contractility and gap junctions in spreading these signals in intact uterine strips, respectively.

3.3 Results

3.3.1 Contractility profiles found in late gestation pregnant rat myometrium

Based on numerous observations it was established that uterine tissue strips could either be spontaneously active or quiescent. Spontaneously active strips could be further divided into two subgroups, strips with regular and stable phasic contractions and those showing irregular contractions (Figure 3.3.1.2 & 3.3.1.3).

Quiescent uterine strips readily responded with an elevation of $[Ca^{2+}]_i$ and force to brief (40 s) application of high-K^+ (40mM KCl) depolarising solution. High-K^+ induced a sustained increase in $[Ca^{2+}]_i$ superimposed by Ca^{2+} spike oscillations which was associated with a concomitant production of force. Removal of high-K^+ resulted in restoration of resting $[Ca^{2+}]_i$ and relaxation of force (Figure 3.3.1.1).

The spontaneously active uterine strips with irregular activity showed a large variation in the number of Ca^{2+} spikes which could easily be distinguished with each spike correlating well with a brief phasic contraction. Typical example is shown in figure 3.3.1.2 which demonstrates an extreme case of irregular activity. Whilst Ca^{2+} spikes and phasic contractions were stereotypical in the tissue samples with irregular type of activity there was great variability in the frequency of Ca^{2+} spikes and contractions which were always submaximal in amplitude due to low frequency of Ca^{2+} spikes (data not shown).

Spontaneously phasic active tissue reached stable activity within 90 minutes and once stabilised, the amplitude, duration and frequency were maintained throughout the rest of the experiment. Average amplitude of spontaneous contractions was 68.94 ± 3.46 % of peak

high-K^+, with the average duration of 14.67 ± 2.35 s and a frequency of 4.98 ± 1.66 contractions per 5 minutes. There was a high variability in the amplitude of spontaneous contractions compared to the amplitude of peak high-K^+ contracture, which was taken for 100 %. Contractions were associated with the burst of Ca^{2+} spikes which showed little variability in the amplitude within the complex Ca^{2+} transient. As a rule the frequency of Ca^{2+} spikes was highest at the beginning of each burst and towards the end of the contraction Ca^{2+} spikes slowed and ceased and $[Ca^{2+}]_i$ returned to basal levels followed by relaxation (Figure 3.3.1.3b).

All three contractility patterns, responded to high-K^+ depolarisation. In quiescent and phasic spontaneously active tissue, this usually was initiated by a fast increase in $[Ca^{2+}]_i$, $[Ca^{2+}]_i$ often fell slightly as a result of both voltage- and time- dependent inactivation of L-type VOCC. When high-K^+ solution was replaced with physiological saline solution there was often see a brief burst of rebound action potential mediated Ca^{2+} spikes which result from reactivation of the inactivated voltage gated Ca^{2+} channels during the repolarisation phase caused by restoration of normal K^+ in the bathing solution. Whilst all three contractility profiles differ in the patterns of Ca^{2+} transients, it was evident from these experiments that for all types of strips the Ca^{2+} spikes were a key mechanism controlling the production of force in pregnant rat myometrium.

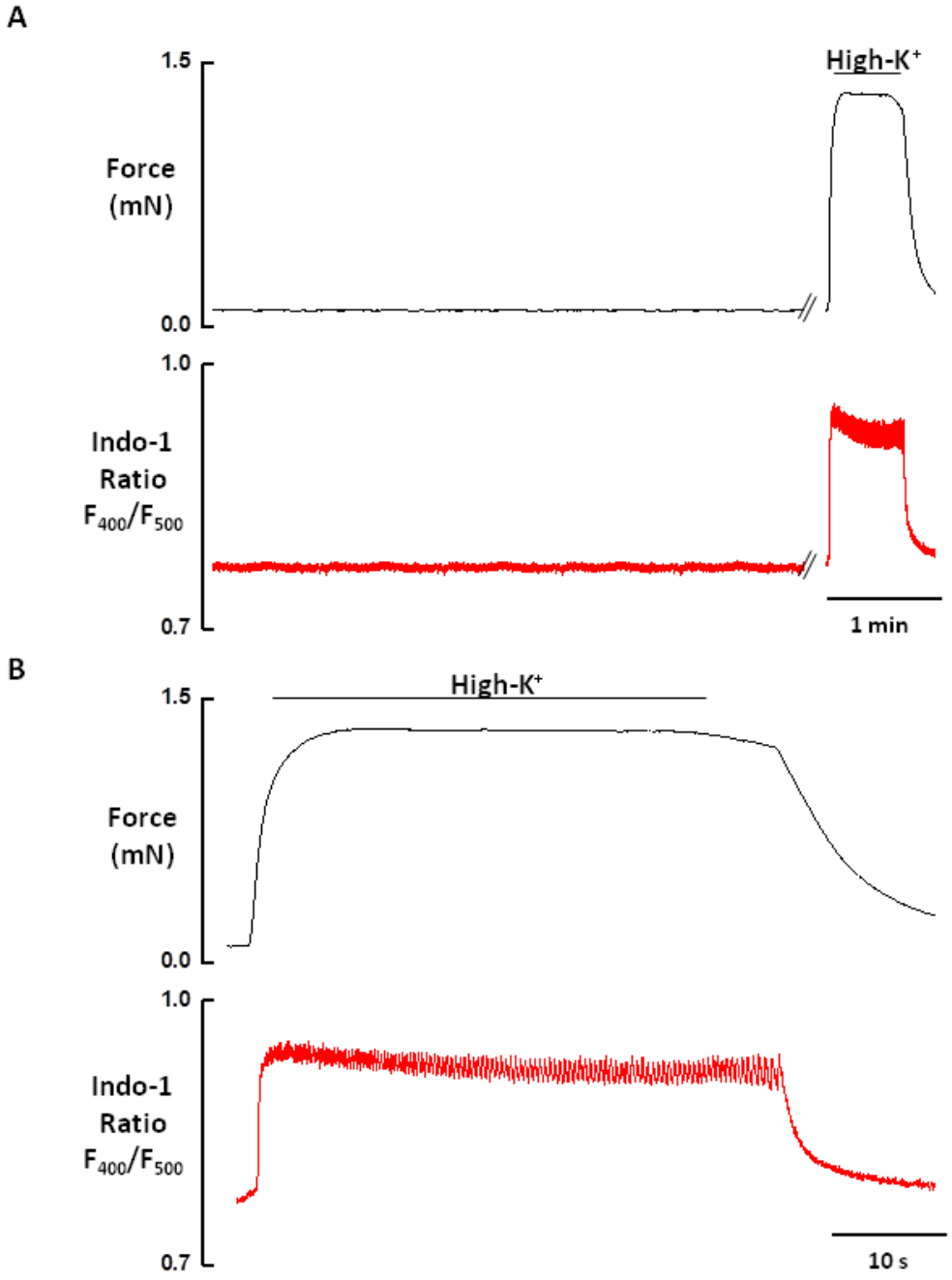

Figure 3.3.1.1 Response of quiescent tissue to high-K $^{+}$ depolarisation.(A) original record of quiescent tissue failing to give spontaneous activity in PSS, but is responsive to high-K^+ (40mM) induced depolarisation, force (black/top trace) and Ca^{2+} transients (red/bottom trace). (B) High-K^+ induced contraction, showing Ca^{2+} spike within the Ca^{2+} transients.

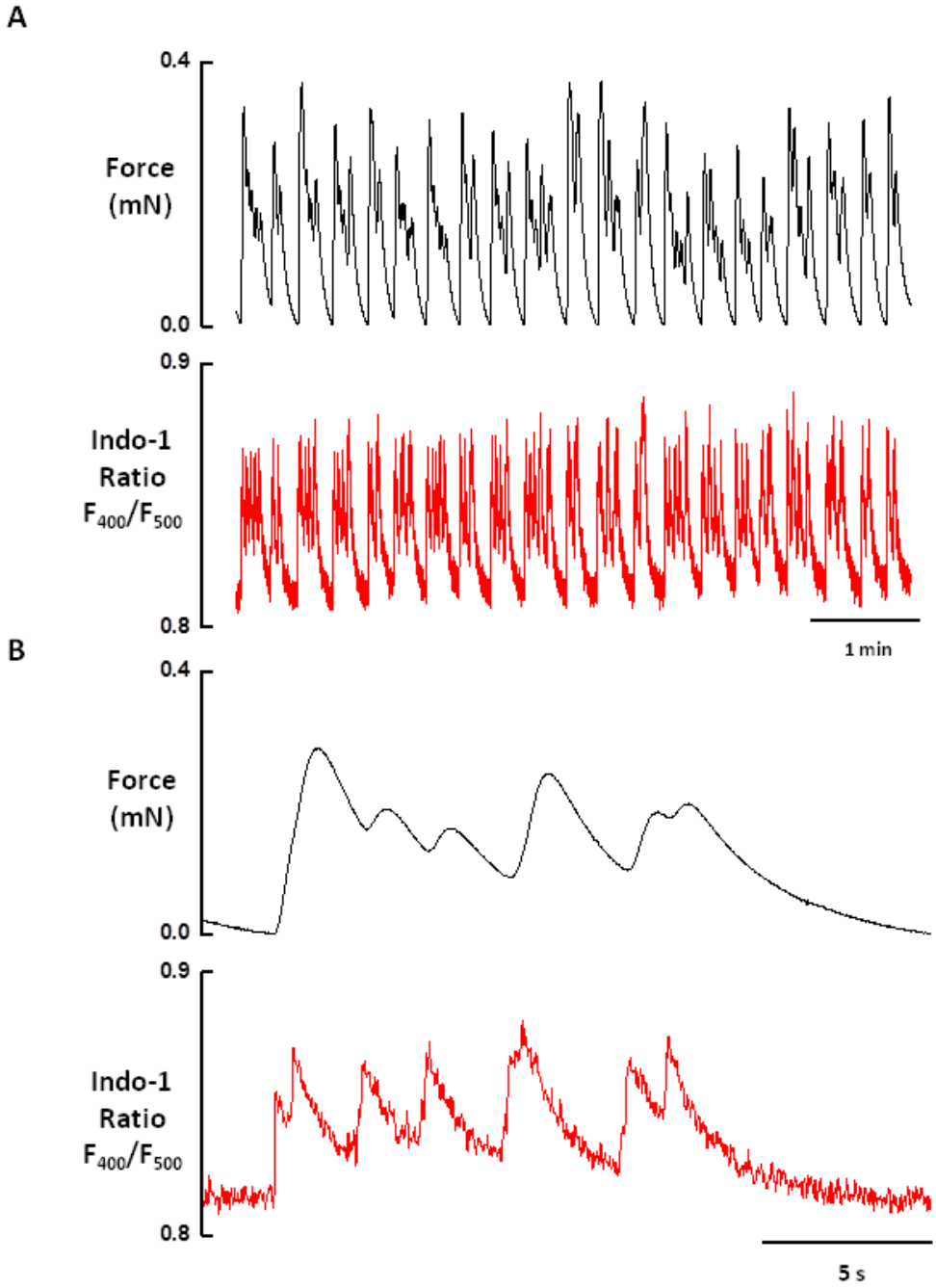

Figure 3.3.1.2 Spontaneous irregular activity in late pregnant rat myometrium. (A) original record of irregular contractility (top trace) and Ca^{2+} transients (bottom trace) in PSS. (B) single contraction and Ca^{2+} transient clearly showing slow Ca^{2+} spikes within the burst of Ca^{2+} spikes resulting in poor summation of phasic contractions.

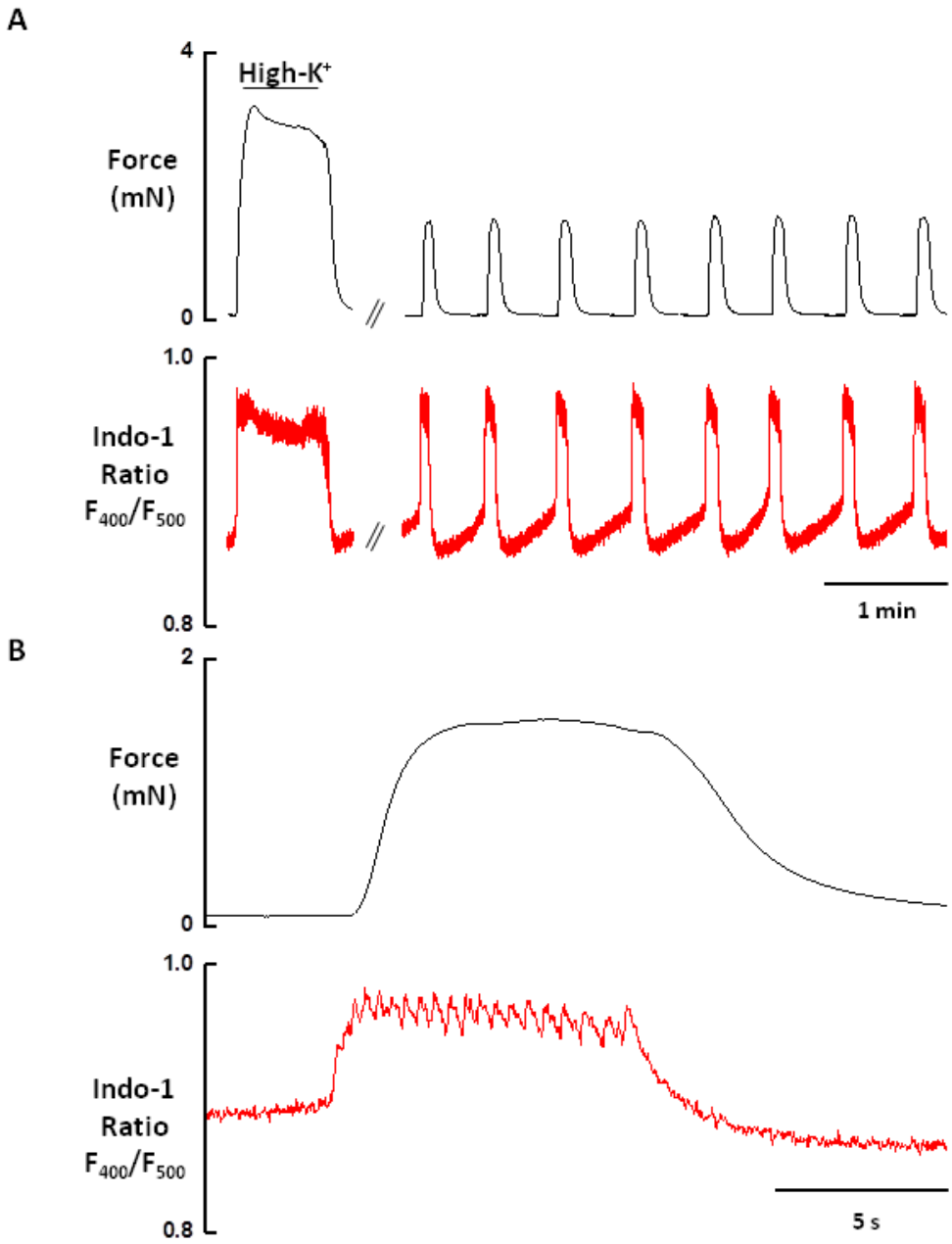

Figure 3.3.1.3 Regular spontaneous phasic activity in late pregnant rat myometrium. (A) Original record of high-K^+ induced and spontaneous contractility (top trace) and Ca^{2+} transients (bottom trace). (B) Single phasic contraction, force (top trace) and Ca^{2+} transients (bottom trace), Ca^{2+} transient showing Ca^{2+} spikes within the burst.

3.3.2 Role of voltage activated Ca^{2+} channels in spontaneous and quiescent myometrial tissue

The role of VOCC in myometrial activity was determined by use of the L-type Ca^{2+} channel inhibitor nifedipine (10μM) (*n* = 5). Addition of nifedipine to spontaneously active tissue resulted in the quick and complete abolishment of both force and Ca^{2+} transients (Figure 3.3.2a). In addition, pre-treatment of spontaneously active or quiescent uterine strips with nifedipine fully blocked high-K induced increase in $[Ca^{2+}]_i$ and force (Figure 3.3.2b). These experiments indicate the key role of VOCC in control of both spontaneous contractions and those induced by high-K^+ depolarisation.

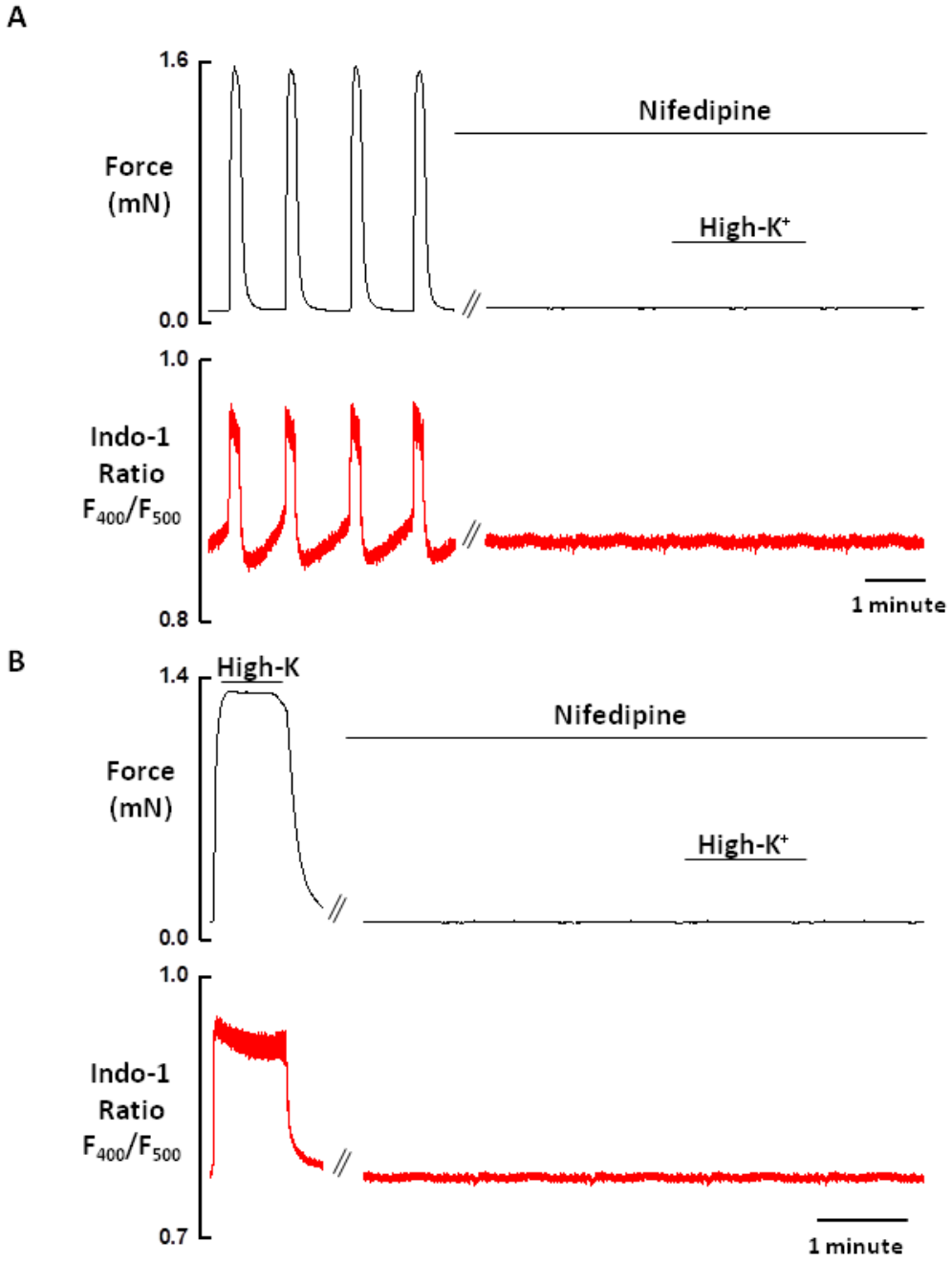

Figure 3.3.2 The effects of VOCC inhibition (10μM nifedipine) on late gestation rat myometrium. Inhibition of L-type Ca^{2+} channels results in the abolishment of force (top traces) and Ca^{2+} transients (bottom traces) in (A) spontaneous activity, and (B) high-K^+ induced contractility in quiescent tissue. (n=5)

3.3.3 The effects of inhibition of gap junctions by 18-β-glycyrrhetinic acid

To investigate the role of gap junctions in control of Ca^{2+} signalling and force in spontaneously active myometrial strips, the effects of the gap junction inhibitor, 18-β-glutherhinic acid (18-β-GA) on Ca^{2+} transient and force in uterine smooth muscle was investigated. These experiments were performed using confocal microscopy combined with force measurements. These experiments showed that inhibition of gap junctions by 18-β-GA resulted in a decrease in the frequency of Ca^{2+} spikes and concurrent decrease in the force of spontaneous contractions, similar to those seen in strips with irregular contractile activity (Figure 3.3.3) (*n*=1). Although it was not regularly studied it was clearly seen that there was a significant decrease in the speed of inter-bundular propagation of the Ca^{2+} spikes which was associated with poor recruitment of the muscle bundles and decrease in the mechanical output (data not shown). While further work is needed to confirm these results, it is evident that gap junctions are needed to propagate Ca^{2+} spikes allowing for the whole tissue to control recruitment and coordination of the mechanical activity of the uterus.

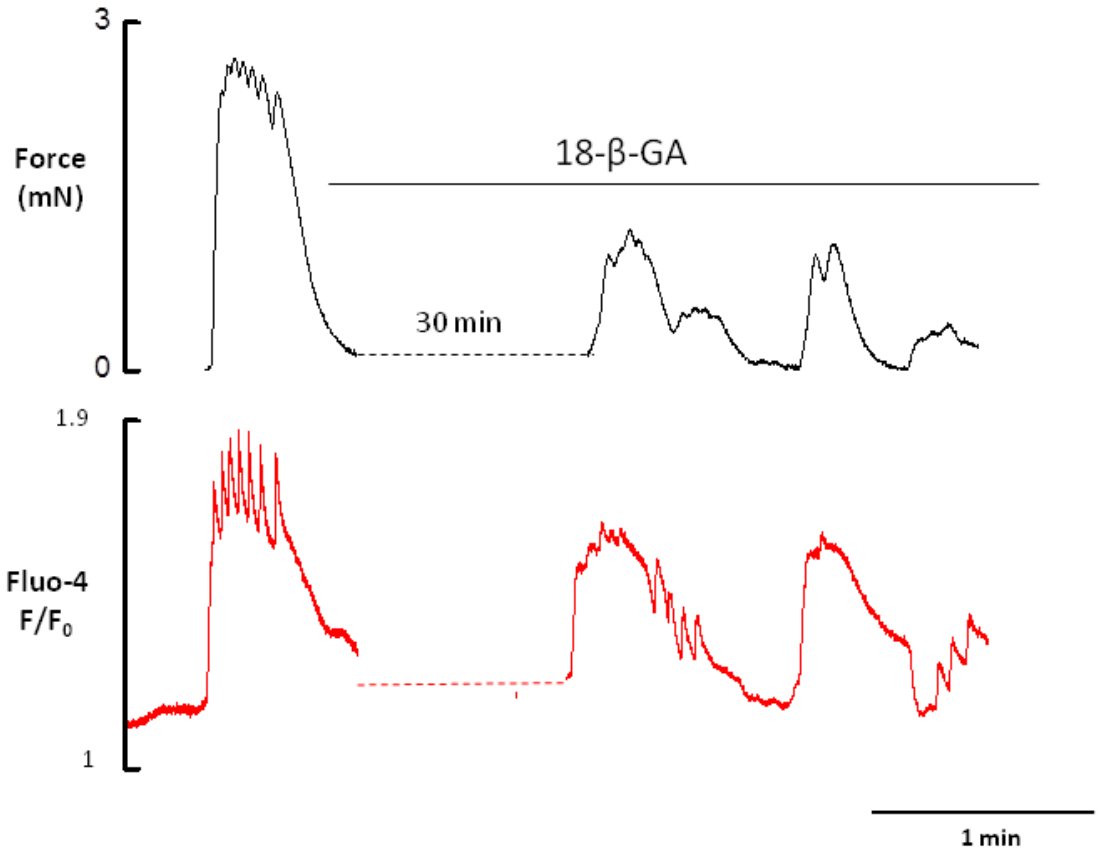

Figure 3.3.3 The effects of gap junction inhibition on myometrial contractility. Simultaneous force and $[Ca^2]_i$ measurements by confocal microscopy at room temperature. Inhibition of gap junctions (50nM 18-β-glycyrrhetinic acid) resulted in a decrease in Ca^{2+} oscillations and concurrent fall in the amount of force produced.

3.4 Discussion

Contractility in uterine smooth muscle is dependent on trains of spike like APs, concurrent Ca^{2+} spikes, caused by Ca^{2+} entry through L-type Ca^{2+} channels, that control the mechanical activity (Mironneau 1973; Shmigol et al. 1998b). Our simultaneous recordings of force and $[Ca^{2+}]_i$ of spontaneous or high-K^+ evoked activity in late gestation pregnant rat myometrium are in good agreement with these early electrophysiological observations. It can be clearly seen that the working unit in uterine smooth muscle is a Ca^{2+} spike which control brief sub-maximal phasic contractions. Clustering of the Ca^{2+} spikes in a burst and intracellular propagation of the Ca^{2+} spikes allows the production of large complex phasic contractions (Figure 3.3.2) (Burdyga et al. 2009)

Spontaneous uterine contraction has been shown to be primarily dependent on influx of Ca^{2+} via L-type VOCC (Shmigol et al. 1998b). This dependence on L-type VOCC could be seen on both spontaneous and quiescent tissue, were the application of the L-type Ca^{2+} channel inhibitor nifedipine resulted in the complete abolishment of both spontaneous and high-K^+ induced Ca^{2+} transient and force.

While the production of force is dependent on the frequency of action potentials and therefore the frequency of Ca^{2+} spikes, it is also dependent on the propagation of the signal through the tissue (Lammers et al. 1994). The better synchronised the tissue is, the more myometrial bundles will be recruited to contribute to the contraction, thus increasing the capacity of force that is able to be produced. The propagation of contractions is due to the presence of gap junctions (Lammers et al. 1994). Inhibition of gap junctions by 18-β-GA resulted in a loss of synchronisation of Ca^{2+} signalling among the muscle bundles which resulted in a decrease in force production due to a decrease in the number of bundles

recruited during a contraction, reinforcing the importance of myometrial gap junctions in the normal functioning of uterine smooth muscle.

These properties of myometrial contractility can account for the three types of spontaneous activity found in pregnant rat longitudinal smooth muscle; normal phasic activity, irregular activity and quiescent activity. Perhaps the easiest way to explain the quiescence in strips which responded to high-K^+ depolarisation was lack of pacemaker activity within the small strip. Lack of pacemaker is most likely responsible for the majority of quiescent samples, were high-K^+ stimulation caused a normal response. Under the influence of high-K^+ induced depolarisation, pacemaker activity is not needed as depolarisation induced by high-K^+ always reaches threshold of activation of VOCC in all bundles (Jmari et al. 1986), and there is no need for the Ca^{2+} signal to propagate. Thus, irrespective of its initial activity all uterine strips respond to high-K^+ stimulation with maximal response.

Irregular activity, similar to quiescent tissue, can be either due to low electrical activity or poor electrical communication between the muscle bundles. Unlike quiescent tissue, it is more likely to be due to asynchronous activity. Frequency of Ca^{2+} spikes representing individual action potentials was very low and could easily be distinguished, which correlated well with brief phasic contractions. The low frequency of Ca^{2+} spikes were responsible for the irregular and low amplitude contractions which was similar to the effects of the gap junction inhibitor 18-βGA.

Compared to quiescent tissue and tissue with irregular activity, spontaneously active tissue has normal pacemaker activity and is well synchronised, to give regular and spontaneous contractions. In this tissue it can be clearly demonstrated, as shown in figure 3.3.1.3 that while force is increased under high-K^+ stimulation the amplitude of the Ca^{2+} transient of

$PGF_{2\alpha}$ induced contraction did not change compared to control, emphasising the importance of frequency of Ca^{2+} spikes being responsible for the amount of force produced (Mironneau 1973; Burdyga et al. 2009). The increase in force seen with high-K^+ stimulation, is probably due to better recruitment of myometrial bundles, higher frequency of Ca^{2+} spikes and sustained rise of $[Ca^{2+}]_i$ compared to control contractions. While this occurs in many cases, it is not universal, those strips with good synchronicity had spontaneous contractions of the same amplitude as high-K^+ induced contractions.

It has been demonstrated that myometrial tissue is able to respond in three characteristic ways under normal physiological experimental conditions, and our data agree and add to the well known mechanisms which control myometrial contractility. After setting up the techniques my next goal was to use current techniques and investigate possible mechanisms involved in stimulant action of $PGF_{2\alpha}$ on Ca^{2+} signalling and force in longitudinal uterine smooth muscle cells.

Chapter 4

The effects of prostaglandin $PGF_{2\alpha}$ on force / Ca^{2+} relationship in pregnant rat myometrium

Chapter 4

The effects of prostaglandin $PGF_{2\alpha}$ on force / Ca^{2+} relationship in pregnant rat myometrium

4.1 Introduction

The importance of temporal and spatial characteristics of $[Ca^{2+}]_i$ in control of spontaneous myometrial contractility has previously been discussed (Chapter 3). A number of possible mechanism have been suggested to be responsible for the increase in myometrial output produced by different agonists which include; a) an increase in $[Ca^{2+}]_i$ (Molnar et al. 1990b), b) an increasing in the frequency of AP/ Ca^{2+} spikes (Burdyga et al. 2009) or c) Ca^{2+} sensitisation (Izumi et al. 1996).

Previous studies have clearly shown that $PGF_{2\alpha}$ increases uterine smooth muscle contractile output in all species studied (Reiner et al. 1976; Tuross et al. 1987; Beretta et al. 2004). The increase in myometrial force seen with $PGF_{2\alpha}$ is dose dependent, whilst sensitivity increases throughout gestation (Crankshaw et al. 1992). The relationship between $[Ca^{2+}]_i$ and force induced by $PGF_{2\alpha}$ is unknown and requires further investigation.

Based on force studies, the importance of both external and intracellular Ca^{2+} in the effects of $PGF_{2\alpha}$ have been shown (Perusquia et al. 1992). The importance of Ca^{2+} signalling was validated with the measurement of $[Ca^{2+}]_i$, also suggesting that both intracellular and extracellular Ca^{2+} are required for the full response of $PGF_{2\alpha}$ (Ruttner et al. 2002). Whilst the importance of Ca^{2+} to the full effects of $PGF_{2\alpha}$ is in no doubt, there are conflicting reports as

to the mechanism of this, some studies suggest an increase in $[Ca^{2+}]_i$ (Molnar et al. 1990b) while others report it remains unchanged (Woodcock et al. 2006). The importance of Ca^{2+} has not been resolved due to the limited investigations of simultaneous force and $[Ca^{2+}]_i$ (Phillippe et al. 1997; Parkington et al. 1999). In addition to the confusion remaining over the relationship between $[Ca^{2+}]_i$ and force, Ca^{2+} sensitisation has also been suggested to be, at least part of the mechanism behind $PGF_{2\alpha}$ (Izumi et al. 1996; Woodcock et al. 2006).

Prostaglandins, especially $PGF_{2\alpha}$ are known to have major roles in pregnancy and labour. $PGF_{2\alpha}$ is a known uterotonic, and inhibition results in delayed parturition (Peri et al. 2002; Kawamata et al. 2008; Goupil et al. 2010). While the functional effects have been thoroughly investigated in a number of species, mechanistic data are limited, especially those directly examining the relationship between force and $[Ca^{2+}]_i$. It is therefore the aim of this study, to examine the effects of $PGF_{2\alpha}$ on Ca^{2+} signalling and force and investigate its possible mechanism.

4.2 Materials and methods

Tissue

Longitudinal myometrial strips (1x 2mm) from the ovarian portion of 22 day gestation Wistar rats were used throughout, obtained as described in chapter 2.

Simultaneous measurements of force and $[Ca^{2+}]_i$

Simultaneous measurements of force and $[Ca^{2+}]_i$ was achieved using a photometric system as previously described (see Chapter 2 for details). Tissue was continuously perfused with PSS until stable contractility was achieved or tissue classified as quiescent. After a period of stable contractility and high-K^+ response, 50nM $PGF_{2\alpha}$ was applied for 5 minutes. 50nM $PGF_{2\alpha}$ was determined as optimal concentration based on preliminary non-cumulative concentration response experiment. 50nM $PGF_{2\alpha}$ was determined to be the optimal concentration as it was the lowest concentration to give a significant increase in phasic contractility; amplitude, duration and frequency.

Analysis and statistics

Analysis was achieved in Origin 8.6. The following force and Ca^{2+} parameters were measured; amplitude, duration, frequency and baseline, over the five minutes treatment time compared to five minutes control contractions. For force, amplitude was taken at 50 % duration and expressed as a percentage of peak high-K^+, duration measured at 50 % amplitude, frequency was the number of contractions within the five minute treatment period and basal force as the average baseline in the 5 minutes. Ca^{2+} was measured similar to force, except for amplitude of Ca^{2+} which due to photobleaching, was measured as the

last 2 contractions in the control period to the first 2 $PGF_{2\alpha}$ contractions, while frequency of Ca^{2+} spikes was determined for the initial 10 seconds of contractions.

Distribution of data was determined by use of Shapiro-Wilk test. Paired t-test or Wilcoxon-signed rank test were used to determine the effect of $PGF_{2\alpha}$. Data is expressed as mean ± S.E.M. or median ± I.Q.R. as appropriate.

4.3 Results

4.3.1 The effects of prostaglandin $PGF_{2\alpha}$ on force / Ca^{2+} relationship on spontaneously active pregnant rat myometrium

The effects of $PGF_{2\alpha}$ were investigated on indo-1 loaded longitudinal myometrial strips from late gestation rat which were either spontaneously active or quiescent. On spontaneously active tissue $PGF_{2\alpha}$ resulted in two responses; either an increase in phasic contractility or a sustained rise in force for the period of agonist application (Figure 4.3.1.1 & 4.3.1.2).

$PGF_{2\alpha}$ increased phasic activity in 7 out of 12 spontaneously active samples (Figure 4.3.1.1). Amplitude of contractions increased from 72.27 ± 4.83 % of peak high-K^+ response to 98.39 ± 5.420 % (p=0.001); duration from an average of 12.23 ± 4.78 s to 16.54 ± 11.61 s (p=0.028) and frequency from 4.98 ± 1.66 contractions / 5 min to 7.19 ± 1.44 contractions (p=0.003). Additionally there was an increase in basal force upon application of $PGF_{2\alpha}$, increasing from 1.48 ± 2.25 % of peak high-K^+ to 4.79 ± 6.83 % (p=0.018) (Figure 4.3.1.1).

It has previously been shown for uterine smooth muscle that contractions are the result of changes in $[Ca^{2+}]_i$ (Longbottom et al. 2000; Burdyga et al. 2009), it is therefore important to investigate changes in $[Ca^{2+}]_i$ and how they correlate with force in the presence of $PGF_{2\alpha}$. As expected each phasic contraction had an associated Ca^{2+} transient. As with force, the duration and frequency of the Ca^{2+} transients increased with the addition of $PGF_{2\alpha}$, duration increased from 15.80 ± 4.43 s to 21.32 ± 5.49 s (p=0.0019), whilst frequency increased from 4.97 ± 0.67 to 7.20 ± 0.58 Ca^{2+} transients / 5 min (p=0.003). Additionally there was also a

slight increase in basal $[Ca^{2+}]_i$, from 0.00 ± 0.001 % of peak high-K^+ to 0.013 ± 0.02 % (p=0.018) (Figure 4.3.1.1). Importantly while there was an increase in the force produced by the tissue, this did not correlate with an increase in the amplitude of the Ca^{2+} transient which remained the same 75.24 ± 12.77 % of peak high-K^+ in control vs 78.12 ± 13.76 % in $PGF_{2\alpha}$ (p=0.197). Ca^{2+} spikes could be well resolved in 6 of the 7 spontaneous samples. The experiments showed that there was an increased in the frequency of Ca^{2+} spikes from 23.83 ± 4.73 spikes (measured during the initial 10 seconds) to 29.16 ± 6.43 spikes in the presence of $PGF_{2\alpha}$ (p=0.038) (Figure 4.3.1.1a(iii)). This data suggest that the frequency of Ca^{2+} spikes within the burst are responsible for the increase in force produced by $PGF_{2\alpha}$.

In 5 samples, $PGF_{2\alpha}$ caused a sustained rise in force from 61.66 ± 5.93 % of peak high-K^+ to 90.53 ± 8.08 % (p=0.053), which remained elevated for the duration of $PGF_{2\alpha}$ application. Again amplitude of the Ca^{2+} transient did not change being 100.63 ± 6.44 % of peak high-K^+ in the absence and 97.15 ± 5.15 % in the presence of $PGF_{2\alpha}$ (p=0.469). After removal of $PGF_{2\alpha}$ tissue returned to control levels (Figure 4.3.1.2).

As with previous results the data obtained suggests that the frequency of Ca^{2+} spikes are responsible for the increased production of force produced by $PGF_{2\alpha}$.

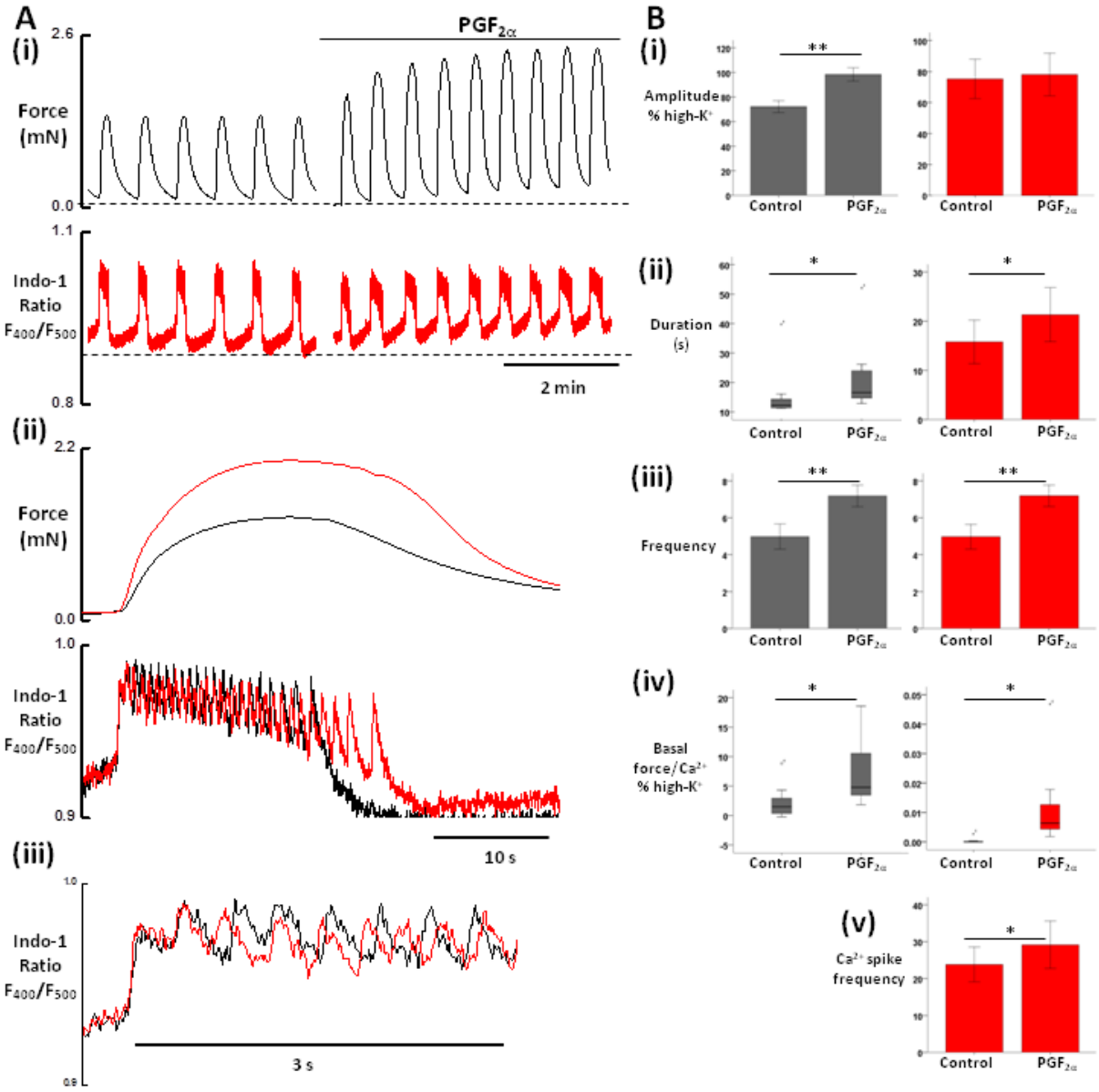

Figure 4.3.1.1 The effects of $PGF_{2\alpha}$ on spontaneously active late gestation rat myometrium. (Ai) Original recording of phasic force (top trace) and Ca^{2+} transients (bottom trace) in the presence of PSS and 50nM $F_{2\alpha}$ (Aii) Superimposed records for a single contraction; PSS (black), $PGF_{2\alpha}$ (red). (Aiii) Ca^{2+} transient for the beginning 3 seconds of spontaneous (black) and $PGF_{2\alpha}$ (red) induced Ca^{2+} transient, showing no change in $[Ca^{2+}]i$, but an increase in the frequency of Ca^{2+} spikes within the burst. (B) Graphs showing force (black) and Ca^{2+} (red) for spontaneous activity compared to $PGF_{2\alpha}$ activity, for; (i) amplitude % high-K^+, (ii) duration, (iii) frequency, (iv) basal force and Ca^{2+} as % high-K and v) frequency of Ca^{2+} spikes for the beginning 10 s of contractions. (n=7 , *p<0.05, **p<0.005)

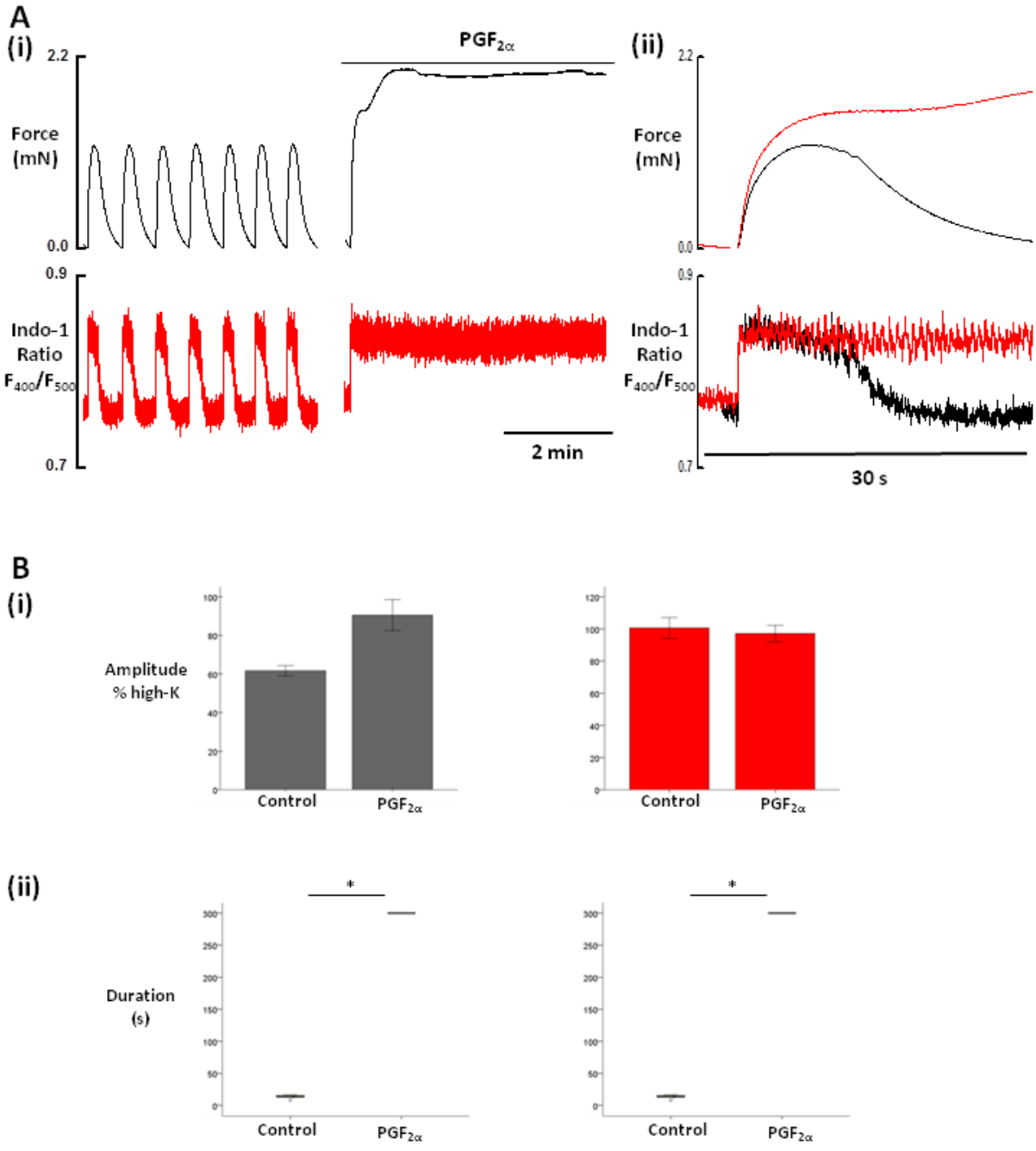

Figure 4.3.1.2 The effects of $PGF_{2\alpha}$ on spontaneously active late gestation rat myometrium. (A) Original recording of spontaneously activie tissue in the presence of PSS and $PGF_{2\alpha}$(50nM), $PGF_{2\alpha}$ induced a sustained contraction for the period of application, force (top trace) and Ca^{2+} transient (bottom trace). (ii) Superimposed records for a single contraction; PSS (black), $PGF_{2\alpha}$ (red). (B) Graphs showing force (black) and Ca^{2+} (red) for spontaneous activity compared to $PGF_{2\alpha}$ activity, for; (i) amplitude % of peak high-K^+ and (ii) duration. (n=5, *p<0.05).

4.3.2 The effects of $PGF_{2\alpha}$ on myometrial strips with irregular activity

$PGF_{2\alpha}$ increased force in spontaneously active phasic tissue by increasing the frequency and duration of Ca^{2+} bursts and the frequency of Ca^{2+} spikes within the burst. In tissue with irregular activity, $PGF_{2\alpha}$ caused an increase in the amplitude and duration of contractions, while $[Ca^{2+}]_i$ mirrored duration it did not show an increase in amplitude (Figure 4.3.2). From the expanded time scale shown in Figure 4.3.2b it is clearly seen that while there is no change in the amplitude of Ca^{2+} spikes there is an increase in the frequency of the Ca^{2+} spikes. It is this increase in frequency that is responsible for the increase in force seen with the addition of $PGF_{2\alpha}$, due to a more efficient summation of the individual phasic contractions.

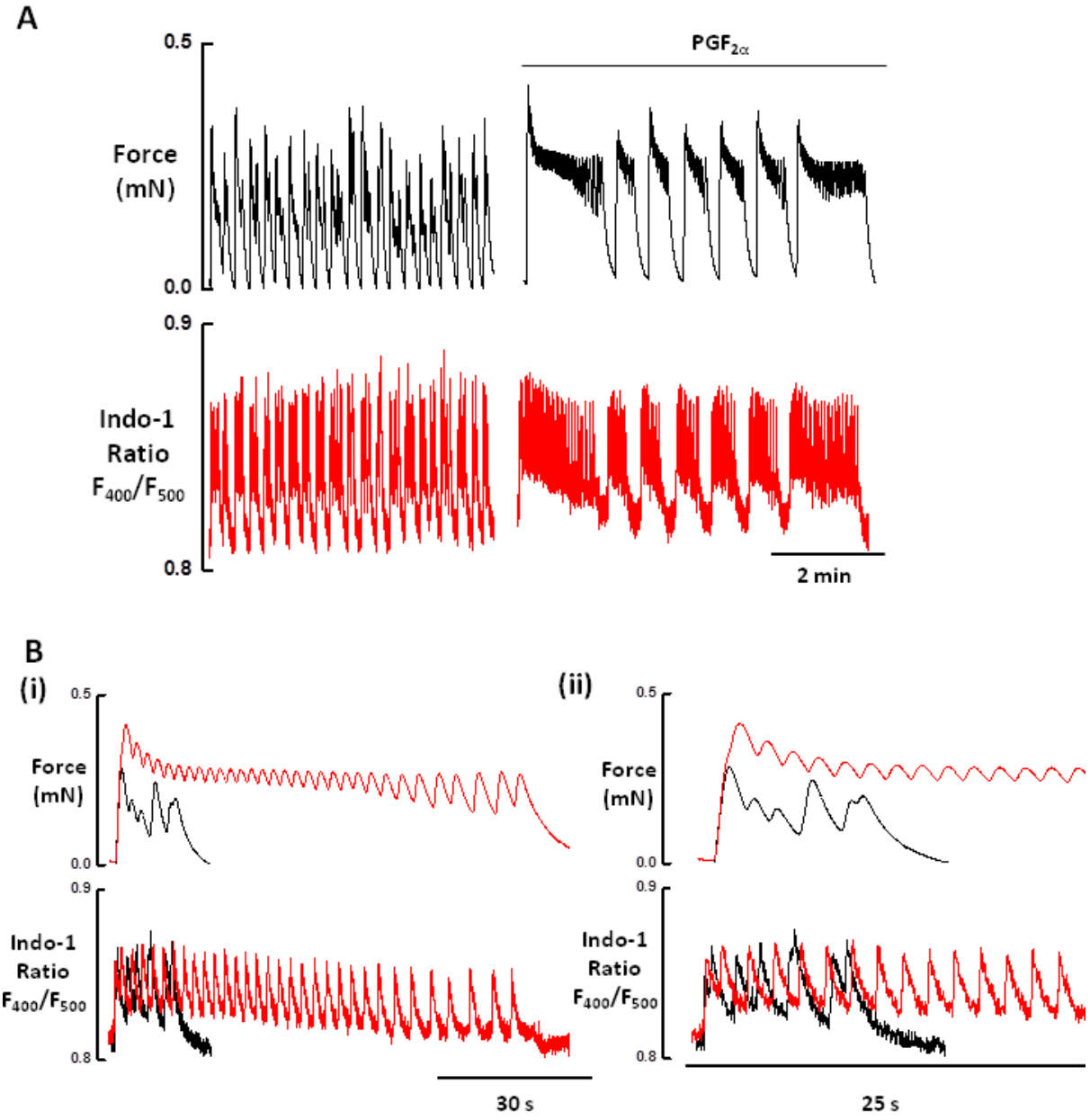

Figure 4.3.2 The effects of $PGF_{2\alpha}$ on spontaneous irregular activity in late gestation rat myometrium. (A) Original recording of broken activity in the presence of PSS and 50nM $F_{2\alpha}$ force (top trace) and Ca^{2+} transients (bottom trace). (B) Superimposed records for a single contraction; PSS (black), $PGF_{2\alpha}$ (red) for force (top trace) and Ca^{2+} transient (bottom trace) (ii) extended time scale, showing increase in Ca^{2+} spike frequency.

4.3.3 The effects of $PGF_{2\alpha}$ on quiescent myometrial tissue

On quiescent tissue which was not spontaneously active but responsive to high-K^+ stimulation, $PGF_{2\alpha}$ caused the initiation of spontaneous phasic activity in 5 out of 6 strips tested. As with other tissue samples Ca^{2+} mirrored force in regards to duration and frequency, but again there was no change in the amplitude of Ca^{2+} spikes despite progressive increase in the amplitude of force seen in the presence of $PGF_{2\alpha}$ (Figure 4.3.3). Upon removal of $PGF_{2\alpha}$, spontaneous activity was stopped and tissue strips were responsive only to high-K^+ stimulation.

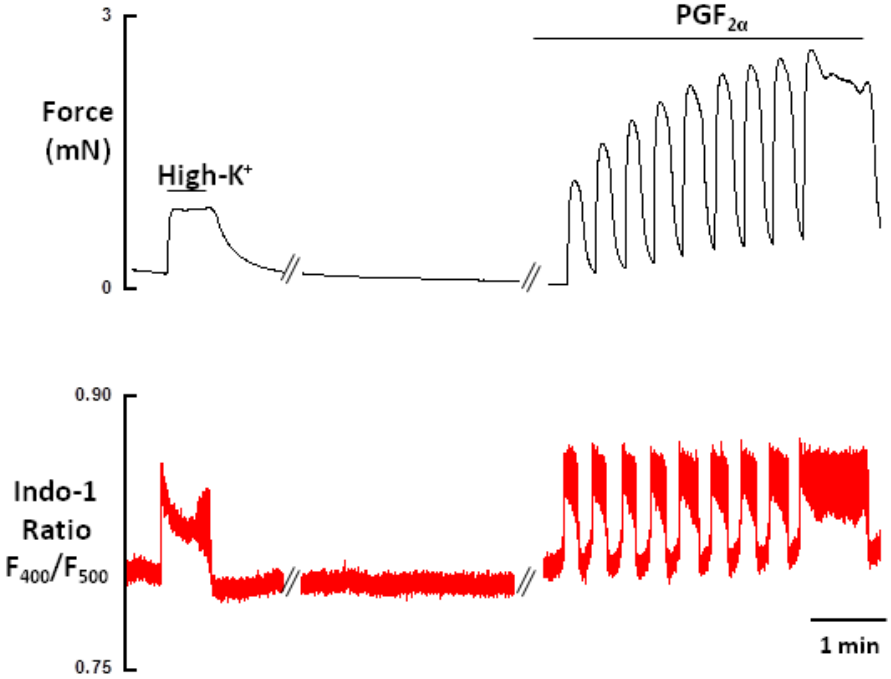

Figure 4.3.3 The effects of $PGF_{2\alpha}$ on quiescent tissue. Original recording of quiescent tissue, but responsive to high-K^+ induced depolarisation. $PGF_{2\alpha}$ results in the initiation of both Ca^{2+} transients and the production of force. Force (top trace) and Ca^{2+} transient (bottom trace). Note that with increasing force of contraction $[Ca^{2+}]_i$ remains the same.

4.3.4 The effects of brief application of $PGF_{2\alpha}$ are long lasting

Agonist have different receptor binding characteristics, $PGF_{2\alpha}$ has previously been shown to have a low dissociation constant (Coleman et al. 1994), it remains bound to its receptor for a long period before it will dissociate and stop exerting its effects. This can be clearly seen in figure 4.3.4, showing $PGF_{2\alpha}$ response and washout period for spontaneous and quiescent tissue, respectively. Whilst the period of time taken for the tissue to return to pre-application contractility profiles is varied, quiescent tissue was usually quickest to stop contracting, whilst tissue producing sustained contractions took the longest to return to control activity.

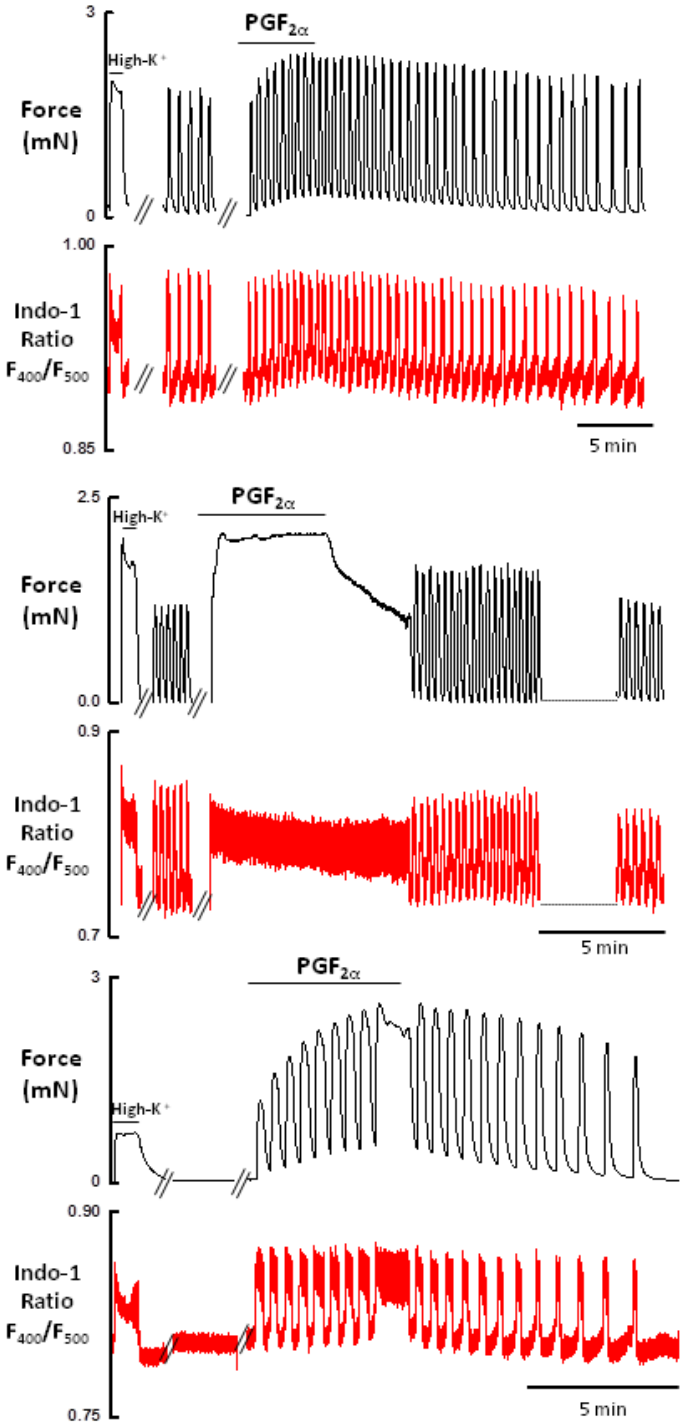

Figure 4.3.4 The effects of brief application of $PGF_{2\alpha}$ are long lasting. Example original recordings of the long lasting effects of a brief 5 min application of $PGF_{2\alpha}$ on (A) spontaneously active tissue giving enhance phasic response, (B) spontaneously active tissue giving sustained response and (C) quiescent tissue. Force (top traces) and Ca^{2+} transient (bottom traces).

4.4 Discussion

There are multiple myometrial agonists, all working to increase force, whether that be an increase in amplitude of force, duration, frequency or combination of all. $PGF_{2\alpha}$ is not similar and in accordance with previous reports caused an increase in frequency, duration and amplitude of contractions (Reiner et al. 1976; Tuross et al. 1987; Beretta et al. 2004; Griffiths et al. 2006) which was seen in this study on uterine strips with three patterns of activity.

There are multiple mechanisms by which uterine agonists increase myometrial contractility. It is generally agreed that agonists work through three principal mechanisms; 1) increase in the amplitude of $[Ca^{2+}]_i$ above that seen during spontaneous activity (Molnar et al. 1990b) 2) depolarisation leading to an increase in frequency of action potentials and thus Ca^{2+} spikes (Burdyga et al. 2009) or 3) an increase in Ca^{2+} sensitisation, whereby there is an increase in the amount of force produced without a change in Ca^{2+} parameters (Izumi et al. 1996).

Previous studies investigating the mechanisms of $[Ca^{2+}]_i$ induced by $PGF_{2\alpha}$, reported that the effect is caused by an increase in $[Ca^{2+}]_i$, that is both intracellular- and extracellular-dependent (Kawarabayashi et al. 1997; Luckas et al. 1999; Ruttner et al. 2002). This correlates well with our observations seen in both spontaneously active and quiescent tissue. However, previous studies showed contradictory results to the effects of $PGF_{2\alpha}$ on Ca^{2+} signalling. Two studies based on human myometrial tissue suggest an increase in the amplitude of the Ca^{2+} transient above that of spontaneous Ca^{2+} transients, was responsible for the increase in force (Molnar et al. 1990b; Parkington et al. 1999) while data obtained on rat longitudinal myometrial strips reported no change in $[Ca^{2+}]_i$, but an increase in basal $[Ca^{2+}]_i$ during $PGF_{2\alpha}$ response (Phillippe et al. 1997). This last study is what we see in rat

tissue, that while duration and frequency of Ca^{2+} transients correlate with force, the amplitude of $[Ca^{2+}]_i$ does not change from that seen with spontaneous contractility, although there was an increase in basal $[Ca^{2+}]_i$, which correlated with the increase in basal force. The principal determining factors in the discrepancies between Ca^{2+} data may be due to technical errors, for example low sampling rate which will not allow Ca^{2+} signals to be accurately resolved or poor synchronisation between the muscle bundles within the strip (e.g. samples with irregular activity, see Figure 4.3.2) and / or movement artefacts present in large preparations which will affect the quality of the fluorescent signals. Fast confocal imaging of Fluo-4 loaded uterine strips was used to verify the temporal and saptail characteristics of the Ca^{2+} transients and correlate it with force. The data obtained strongly suggest that in well synchronised strips which show regular activity (e.g. Figure 3.3.1.3) there was a good correlation between the frequency of Ca^{2+} spikes and the amplitude of force with no change in the amplitude of the Ca^{2+} spikes. Electrophysiological experiments also showed that each spike was associated with a brief action potential of constant amplitude which again suggests that the amplitude of Ca^{2+} spikes is expected to be constant. The mechanism of Ca^{2+}-induced Ca^{2+} release which could amplify a signal in uterine smooth muscle is is not present. Overall the data obtained strongly suggest that contraction of uterine strips is produced by a burst of action potential mediated Ca^{2+} spikes of constant amplitude which give rise to brief phasic contractions. Summation of these phasic contractions results in a bulid up of the complex tetanic-like phasic contraction similar to that seen in skeletal muscles.

It is clear from our data that whilst $PGF_{2\alpha}$ does not increase the amplitude of Ca^{2+} in rat myometria, it does result in an increase in the frequency of Ca^{2+} spikes within the burst of Ca^{2+} spikes. As previously described in chapter 3, in pregnant rat myometrium, each Ca^{2+} spike is associated with a single spike like action potential (Burdyga et al. 2009) and contractions consist of trains of action potentials which summate to increase force, were the higher the frequency of action potential the greater degree of force produced (Mironneau 1973). Based on this account of myometrial contraction, it can be extrapolated that the increase in Ca^{2+} spikes seen with $PGF_{2\alpha}$, is due to an increase in the frequency of action potentials. These data are in agreement with the electrophysiological observations of other agonists, which caused an increase in the frequency of action potentials within a burst (Kuriyama et al. 1976) and the frequency of bursts of action potentials (Lammers et al. 1999), while inhibitory chemical such as histamine which cause uterine relaxation, reduced it (Blyth 1972).

Whist there are contradictory opinions regarding $[Ca^{2+}]_i$ correlating with the amount of force produced by pregnant rat myometrial tissue, there is little dispute regarding the cause of the increase in frequency and duration of $PGF_{2\alpha}$ induced contractility. $PGF_{2\alpha}$ increased both frequency and duration of both the Ca^{2+} transient and phasic contractions, as seen previously (Phillippe et al. 1997). The production of force is directly correlated to the rise in $[Ca^{2+}]_i$, with the duration and frequency of Ca^{2+} transients correlating directly with the duration and frequency of force. $[Ca^{2+}]_i$ is linked with action potentials, with an influx of Ca^{2+} through VOCC responsible for the upstroke of the action potential (Shmigol et al. 1998b) and so upon cessation of action potentials $[Ca^{2+}]_i$ falls, giving the relationship between the duration of the Ca^{2+} transient and action potential burst.

The single previous study of membrane potential induced by $PGF_{2\alpha}$ on pregnant rat myometrium corroborates the relationship between force and $[Ca^{2+}]_i$, showing a slow depolarisation and an increase in both frequency and duration of action potential bursts (Osa et al. 1983) correlating with the increase in frequency and duration of Ca^{2+} transients and contractility seen in this study.

In investigating the relationship between force and Ca^{2+} in smooth muscle, the role of Ca^{2+} sensitisation cannot be ignored. Other uterine agonists are known to work at least in some part through the effects of Ca^{2+} sensitisation (Izumi et al. 1996). While this study does suggest an increase in the frequency of Ca^{2+} spikes as the cause of increased force seen with $PGF_{2\alpha}$, it does not discount other contributory mechanisms, such as Ca^{2+} sensitisation, or explain the mechanisms behind the increase in Ca^{2+} spikes.

Chapter 5

How $PGF_{2\alpha}$ affects force/Ca^{2+} relationship in uterine smooth muscle

Chapter 5

How $PGF_{2\alpha}$ affects force/Ca^{2+} relationship in uterine smooth muscle

5.1 Introduction

The mechanisms by which $PGF_{2\alpha}$ increase myometrial contractility has yet to be elucidated. From previous studies the importance of Ca^{2+} influx through VOCC and Ca^{2+} release from the SR have been suggested (Parkington et al. 1999; Coleman et al. 2000). Other mechanisms suggested to be involved include; modulation of the Na^{+}/K^{+} ATPase activity (Parkington et al. 1999), Ca^{2+} sensitisation (Izumi et al. 1996) and a reduction in cyclic [AMP] (Goureau et al. 1990).

The upstroke of action potentials and subsequent contraction in rat uterine smooth muscle is dependent upon the gradual depolarisation of the membrane until threshold activation of VOCC is reached, culminating in influx of Ca^{2+} (Shmigol et al. 1998b). Membrane potential is achieved through an unequal distribution of ions across the plasma membrane, which in uterine smooth muscle is primarily set through the electrochemical gradients of Na^{+}, Cl^{-}, K^{+} and Ca^{2+}, maintained by the various channels, pump and transporters within the plasma membrane. Many agonists act to change the permeability of the plasma membrane by modulating one or more ion channels, transporters and pumps. For example a number of endogenous and exogenous ligands are able to exert their effects through BK_{Ca} channels. Both adenylyl cyclase (Okawa et al. 2000) and nitric oxide (Okawa et al. 1999) work through opening of the BK_{Ca} channels decreasing contractility, whilst oxytocin increases Cl^{-} current through Cl_{Ca} channels, increasing contractility (Arnaudeau et al. 1994; Jones et al. 2004).

Another principal way in which agonists are able to increase myometrial output, is to release Ca^{2+} from the SR, as a result of GPCR PLC generated IP_3. This mechanism is utilised by a number of agonists including; oxytocin (Molnar et al. 1995; Sanborn 1995), carbachol (Khac et al. 1996; Houdeau et al. 2005) and ATP (Gerwins et al. 1992). In addition to increasing $[Ca^{2+}]_i$, the depletion of the SR can lead to influx of Ca^{2+}, in a mechanism known as store operated Ca^{2+} entry (SOCE), thus further enhancing $[Ca^{2+}]_i$ (Putney 2011). SOCE can be seen in the response of intracellular Ca^{2+} characteristics to agonists. SOCE has been associated with a secondary sustained rise in the $[Ca^{2+}]_i$ in response to agonist (Wayman et al. 1996a; Wayman et al. 1996b; Parekh et al. 2005), while re-admittance of Ca^{2+} after agonist induced store depletion results in a sustained increase in basal $[Ca^{2+}]_i$ which is nifedipine resistant, discounting VOCC involvement, and can be induced by CPA and thapsigargin (SERCA inhibitors), discounting ROC involvement. These characteristics along with use of SOCE inhibitors have identified SOCE as a mechanism of action in many cell types, including uterine smooth muscle (Shlykov et al. 2003; Noble et al. 2009). Unlike other types of smooth muscle, SOCE has only recently been identified in the myometrium, and as such investigations are limited, although both oxytocin (Monga et al. 1999; Fu et al. 2000) and bradykinin (Wassdal et al. 1998) have been identified as possible agonists working through this mechanism in uterine smooth muscle.

In addition to influx of Ca^{2+} through VOCC and SOCE, agonists have also been suggested to result in an influx of Ca^{2+} through ROC. ROC were identified in smooth muscle based on the inward Ca^{2+} current activated upon agonist stimulation. These currents are not voltage sensitive, like L-type Ca^{2+} channels (Murray et al. 1991) although they are also often not Ca^{2+} selective, and are frequently permeable to both Na^+ and K^+ (Benham 1989). Due to this lack

of selectivity, there is overlap between ROC and NSCC; channels activated in response to agonists which allow the passage of different ions (Shimamura et al. 1994; Miyoshi et al. 2004). Confusion regarding the exact nature of ROC and NSCC has recently been exacerbated in smooth muscle, due to the discovery of SOCE. Many uterine agonists work by Ca^{2+} release from the SR, store depletion has been shown to result in the activation of both Ca^{2+} and non-selective inward currents (Hoth et al. 1993; Albert et al. 2003b; Parekh et al. 2005). It is not known whether the inward Ca^{2+}- or non-selective currents induced by many myometrial agonists are due to direct coupling with ion channels, or involved with secondary messengers, such as DAG, which has been shown to directly activate a non-selective inward current (Guinamard et al. 2004) or due to SOCE.

Agonists rarely work by a single mechanism, oxytocin, perhaps the most studied myometrial stimulant, works through at least three mechanisms; 1) an increase in $[Ca^{2+}]_i$ above resting levels (Anwer et al. 1989; Monga et al. 1996) 2) an increase in the frequency of action potentials (Taira et al. 1967; Osa et al. 1973) and 3) Ca^{2+} sensitisation (Tahara et al. 2002; Woodcock et al. 2004; Shmygol et al. 2006; Kawamata et al. 2007). Through its $G\alpha_{q/11}$ coupled-receptor, oxytocin activates PLC (Ku et al. 1995) resulting in an increase in both IP_3 and DAG (Shlykov et al. 2004; Sanborn et al. 2005). IP_3 releases Ca^{2+} from intracellular stores, while DAG has been associated with both Ca^{2+} sensitisation and Ca^{2+} influx through NSCC (TRP channels) (Shlykov et al. 2004), an inward current dependent on both Ca^{2+} and Na^{+} has been identified in response to oxytocin (Shimamura et al. 1994). In addition to increasing $[Ca^{2+}]_i$ through store release, a portion of the increase in $[Ca^{2+}]_i$ upon oxytocin stimulation has been shown to be extracellular dependent, which cannot be accounted solely by the increase in Ca^{2+} flux through L-type Ca^{2+} channels (Sanborn et al. 1998). The

extra Ca^{2+} influx is thought to be through SOCE (Kawamata et al. 2007) although this has not been thoroughly investigated to date.

$PGF_{2\alpha}$ like oxytocin is a myometrial stimulant, working through its G-protein coupled receptor, as such it is likely that the mechanism by which it increases myometrial output is likely to be multifaceted. To date many mechanisms have been suggested, but not investigated fully; for this reason it was decided to investigate the involvement of some of the principal mechanisms of uterine agonists, primarily VOCC, ROC and NSCC in addition to the role of store release and subsequent SOCE.

5.2 Materials and methods

Tissue

Longitudinal myometrial strips (1x2 mm) and isolated myocytes from the ovarian portion of 22 day gestation Wistar rats were used throughout, obtained as described in Chapter 2.

Simultaneous measurements of force and $[Ca^{2+}]_i$

Simultaneous measurements of force and $[Ca^{2+}]_i$ was achieved using a photometric system as previously described (see Chapter 2 for details). Tissue strips were continuously perfused with PSS or PSS 0Ca in the presence or absence of other reagents. Tissue was first left until stable contractility was achieved or tissue classified as quiescent. To determine if modulation of membrane potential was involved in the effects of $PGF_{2\alpha,}$ $PGF_{2\alpha}$ was applied to high-K^+ (40mM KCl) contracting tissue. To investigate the involvement of VOCC, $PGF_{2\alpha}$ was applied to tissue in the presence and absence of nifedipine (10µM). While store release was investigated by firstly maximally filling the SR by a brief (40s) application of high-K^+ (40mM KCl), external Ca^{2+} removed (2mM EGTA) for 3 minutes followed by addition of agonist in the continuing absence of Ca^{2+}. Ca^{2+} was then re-admitted into the external media and rebound parameters in the presence of nifedipine (10µM), and the SOCE inhibitors; La^{3+} (10µM) and Gd^{3+} (10µM) were analysed; using 20µM CPA as a positive control for SOCE. NSCC/ROC was studied using a combination of CPA (20µM) and nifedipine (10µM), to maximally activate SOCE and inhibit L-type VOCC.

Confocal measurement of $[Ca^{2+}]_i$

To characterise $PGF_{2\alpha}$ release from the store, confocal microscopy was used on both myometrial strips and isolated cells in the absence of external Ca^{2+}. For both myometrial strips and isolated myocytes, high-K^+ was applied briefly (40s) to fill the store, followed by

removal of external Ca^{2+} (2mM EGTA) and the addition of 5μM $PGF_{2\alpha}$ in the continued absence of external Ca^{2+}.

Analysis and statistics

Application of $PGF_{2\alpha}$ onto both high-K^+ and CPA induced contractions was analysed by measuring the peak amplitude of high-K^+ / CPA response in the presence of $PGF_{2\alpha}$ compared to the amplitude directly preceding agonist application, normalised to peak amplitude of initial high-K^+ response, taken for 100 percent. A similar technique was used to determine the effects of inhibitors on $PGF_{2\alpha}$ / CPA contracting tissue; amplitude of agonists was compared to amplitude after 2 minutes of inhibitor, expressed as a percentage of peak high-K^+, while amplitude of response to pre-treatment with nifedipine was compared to control experiment.

Store release was measured from baseline at time of agonist application to peak response, expressed as a percentage of peak high-K^+. Rebound amplitudes were taken 2 minutes after re-admittance of Ca^{2+} , or addition of nifedipine, with baseline taken at the time of Ca^{2+} re-admittance, again expressed as a percentage of high-K^+, and compared to appropriate control experiment.

For confocal microscopy experiments the following temporal characteristics were measured; time to peak, the time taken from the beginning of Ca^{2+} rise to the front of Ca^{2+} peak, duration at 50% amplitude, half time of relaxation, the time for the Ca^{2+} transient to fall from the amplitude at 50% duration to 50% amplitude. The speed of propagation of Ca^{2+} wave was also analysed, the time taken for a Ca^{2+} wave to travel between two regions of interest over the distance between the two regions of interest.

Distribution of data was determined by use of Shapiro-Wilk test. Paired t-test or Wilcoxon-signed rank test, or independent samples t-test or Mann-Whitney U test were used to determine significance, with significance set at $p<0.05$. Data is expressed as mean ± S.E.M. or median ± I.Q.R. as appropriate.

5.3 Results

5.3.1 The effects of $PGF_{2\alpha}$ on membrane potential

In uterine smooth muscle, many agonist work by modulating membrane potential. Depolarisation of the membrane ensures that the maximum number of VOCC are open resulting in an influx of Ca^{2+}, increasing $[Ca^{2+}]_i$, allowing for contraction. From preliminary unpublished data (Figure 5.3.1) (Burdyga, T.), it can be seen that $PGF_{2\alpha}$ results in depolarisation and the initiation of a train of action potentials, the train of action potentials result in summation of phasic contractions (seen as notches in the force trace) associated with each action potential, increasing the total force.

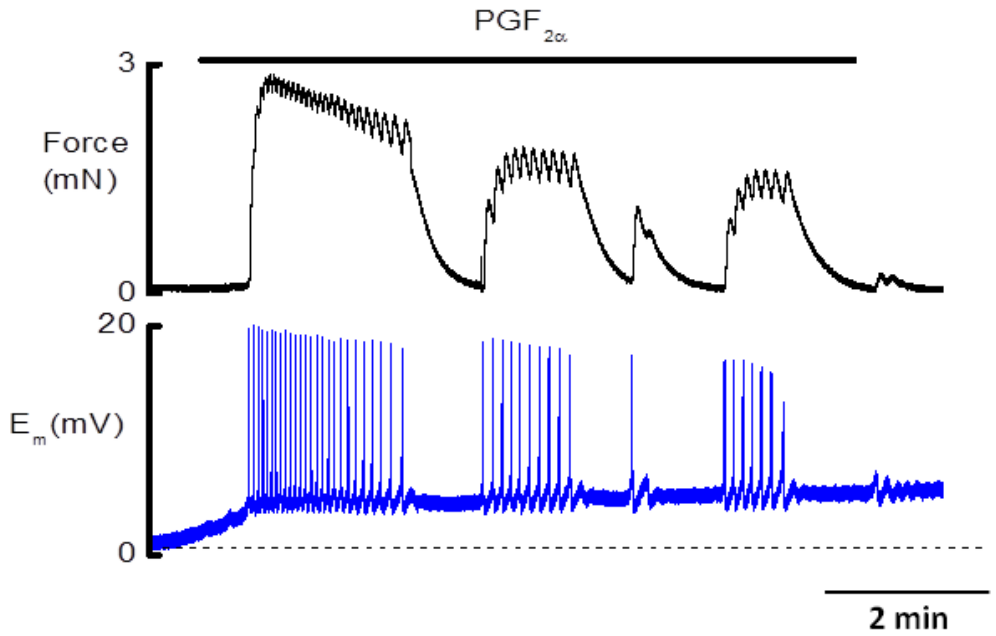

Figure 5.3.1 Effects of $PGF_{2\alpha}$ on membrane potential and force. $PGF_{2\alpha}$ causes depolarisation of the membrane and initiation of trains of action potentials and summation of force. Original trace of electrical acitivity (blue / bottom trace) and force (black / top trace) in the presence of $PGF_{2\alpha}$ (Burdyga, T. unpublished data)

5.3.2 The effects of $PGF_{2\alpha}$ on partially depolarised tissue.

To determine if $PGF_{2\alpha}$ works through membrane depolarisation, $PGF_{2\alpha}$ was applied during the tonic contraction induced by high-K^+ (40mM KCl) induced partial depolarisation. Addition of 50nM $PGF_{2\alpha}$, a concentration previously shown to significantly increase force, resulted in no alteration in either the amplitude of contraction or $[Ca^{2+}]_i$; amplitude of the sustained component of high-K^+ contraction was 72.48 ± 14.44 % of peak high-K^+ compared to 82.27 ± 21.04 % (*n*=5, p=0.372) in the presence of $PGF_{2\alpha}$, and sustained level of $[Ca^{2+}]_i$ was 67.96 ± 6.99 % of peak high-K^+ and 69.29 ± 5.85 % respectively (*n*=5, p=0.374). In addition to no alteration in either force or amplitude of $[Ca^{2+}]_i$, the frequency of Ca^{2+} spikes also remained the same (Figure 5.3.2a).

When a higher concentration of $PGF_{2\alpha}$ (5µM) was used, there was a transient increase in force and $[Ca^{2+}]_i$, with a cessation of Ca^{2+} spikes (Figure 5.3.2b). Force increased from 70.22 ± 10.26 % of peak high-K^+ to 122.80 ± 13.32 % (*n*=5, p=0.015), while $[Ca^{2+}]_i$ increased from 60.80 ± 8.55 % of peak high-K^+ to 88.25 ± 8.98 % (*n*=5, p= 0.011) (Figure 5.3.2b).

The discrepancy between the effects of 50nM and 5µM $PGF_{2\alpha}$ can have two explanations; firstly the photometric apparatus was not sensitive enough to resolve small changes in $[Ca^{2+}]_i$ induced by the lower concentration of $PGF_{2\alpha}$, or that the higher concentration of the agonist was able to recruit additional mechanisms of Ca^{2+} mobilisation. To ensure that all the effects of $PGF_{2\alpha}$ are present, further investigations were achieved with use of a supramaximal concentration of 5µM $PGF_{2\alpha}$.

The increase in $[Ca^{2+}]_i$ and force induced by $PGF_{2\alpha}$ in partially depolarised tissue could be due to Ca^{2+} influx mediated by SOCE or entry through ROC/NSCC, in addition to release from the SR.

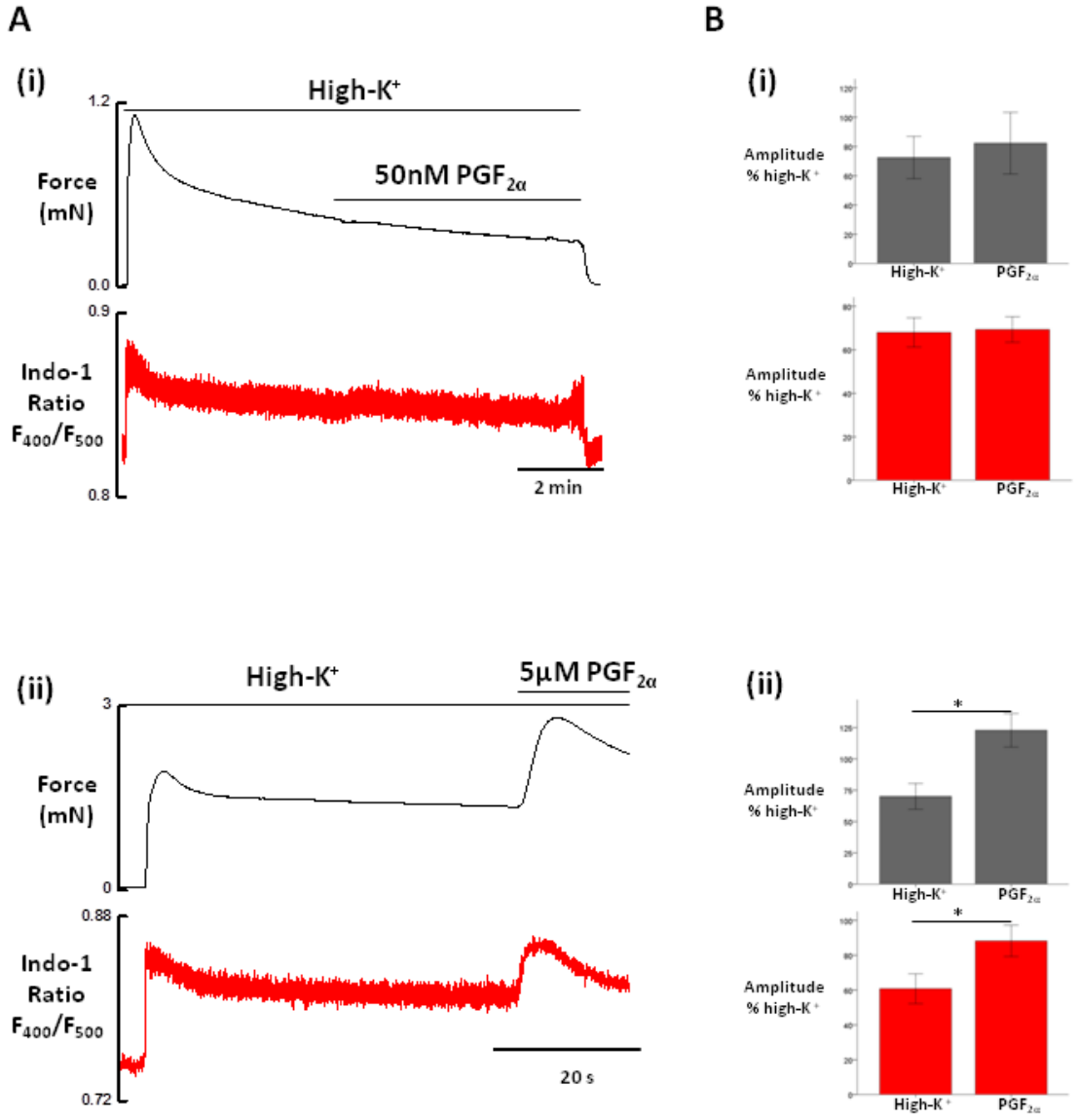

Figure 5.3.2 The effects of $PGF_{2\alpha}$ on high-K^+ partially depolarised myometrial tissue. (A) Original recordings of force (top trace) and Ca^{2+} (bottom trace) in the presence of high-K (40mM KCl) (i) 50 nM $PGF_{2\alpha}$ did not effect either force or $[Ca^{2+}]_i$, whilst (ii) 5 µM $PGF_{2\alpha}$ resulted in a transient increase in both force and $[Ca^{2+}]_i$. (B) Graphs showing force (black) and Ca^{2+} (red), for; (i) 50 nM $F_{2\alpha}$ and (ii) 5 µM $F_{2\alpha}$ compared to pre-$PGF_{2\alpha}$ levels. (n=5, *p<0.05)

5.3.3 The effect of L-type VOCC inhibition.

We have previously found that $PGF_{2\alpha}$ resulted in an influx of Ca^{2+} and the production of force. In order to investigate the possible contribution of plasma membrane ion channel enhancement of Ca^{2+} flux through VOCC we used nifedipine to inhibit L-type VOCC. Application of 10μM nifedipine inhibited high-K^+ induced contractions in pregnant rat myometrial strips, confirming inhibition of L-type Ca^{2+} channels. Application of supramaximal concentration of $PGF_{2\alpha}$ (5μM) in the continuing presence of nifedipine resulted in an increase in $[Ca^{2+}]_i$, and the production of force for the duration of treatment. Amplitude of force increased to 20.88 ± 27.98 % of peak high-K^+ (*n*=5, p=0.005) and $[Ca^{2+}]_i$ increased to 74.65 ± 20.68 % (*n*=5, p=0.005) (Figure 5.3.3.1), whilst there was an increase in $[Ca^{2+}]_i$, it is important to note that there were no Ca^{2+} spikes within the Ca^{2+} transient, unlike during normal $PGF_{2\alpha}$ response.

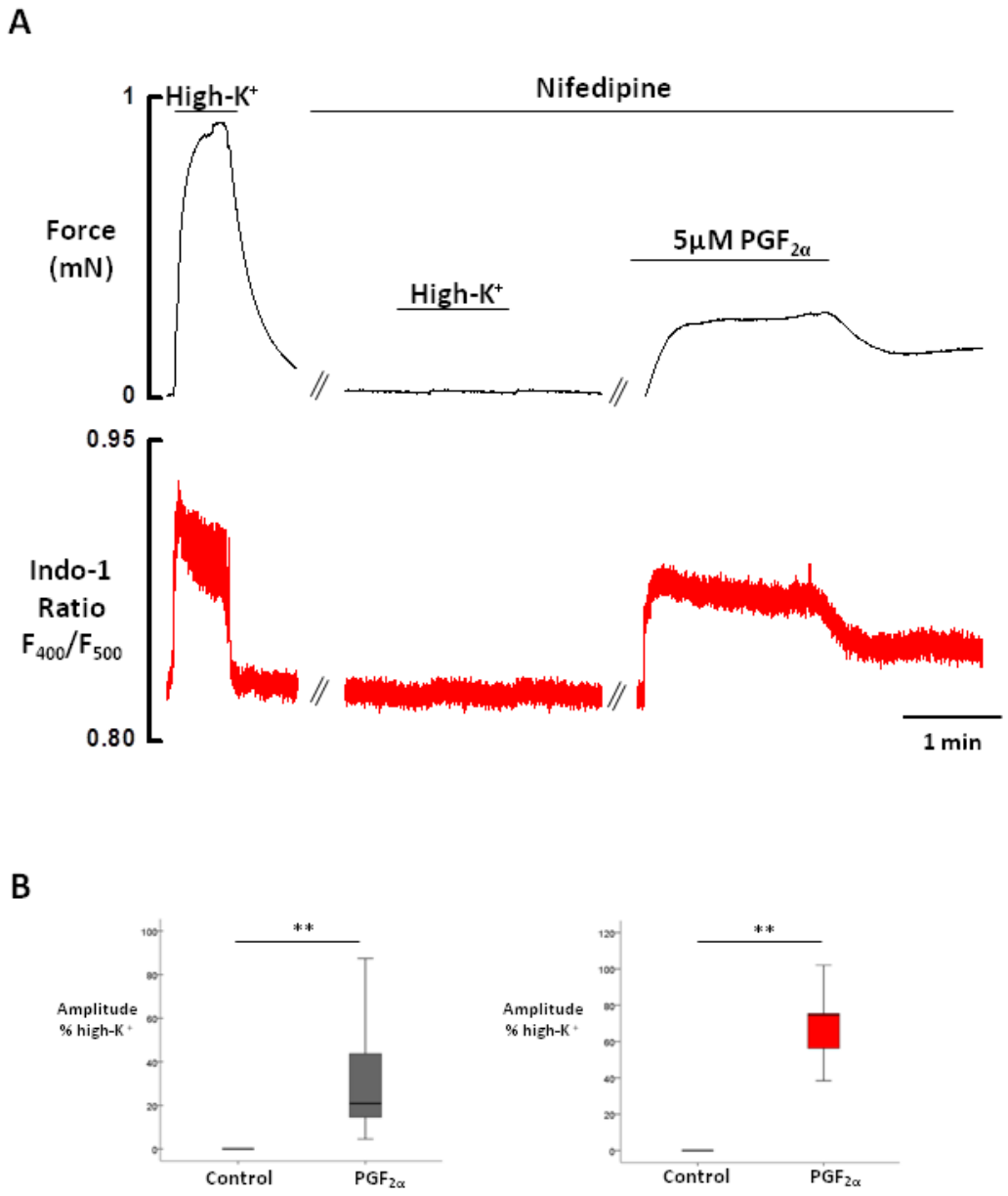

Figure 5.3.3.1 The effects of L-type VOCC inhibition (10µM nifedipine) on $PGF_{2\alpha}$ response in late gestation myometrial tissue. Application 5 µM $PGF_{2\alpha}$ resulted in the generation of force and an increase in $[Ca^{2+}]_i$ in the absence of L-type VOCC. (A) Original recordings of force (top trace) and $[Ca^{2+}]_i$ (bottom trace) in response to high-K^+ (40 mM KCl), nifedipine (10 µM) and 5 µM $F_{2\alpha}$ in the continuing presence of nifedipine (B) Graphs showing force (black) and Ca^{2+} (red), for $PGF_{2\alpha}$ compared to control. (n=5, **p<0.005)

Similar to nifedipine pre-treatment, when L-type VOCC were inhibited during $PGF_{2\alpha}$ induced contraction, there was a reduction in both force and $[Ca^{2+}]_i$, but neither were completely abolished. Force fell from 104.46 ± 12.34 % of peak high-K^+ to 23.88 ±8.24 % (*n*=5, p=0.000), correlating to a 78.90 % reduction, while $[Ca^{2+}]_i$ fell from 38.77 ± 7.89 % of peak high-K^+ to 24.82 ±5.71 % (*n*=5, p=0.036), a 14.01 % reduction (Figure 5.3.3.2). Importantly to note as with the previous nifedipine experiment, inhibition of L-type Ca^{2+} channels again resulted in the abolishment of Ca^{2+} spikes.

From these experiments it is clear that there is a voltage-independent Ca^{2+} entry resistant to nifedipine, increasing $[Ca^{2+}]_i$ and producing force induced by $PGF_{2\alpha}$.

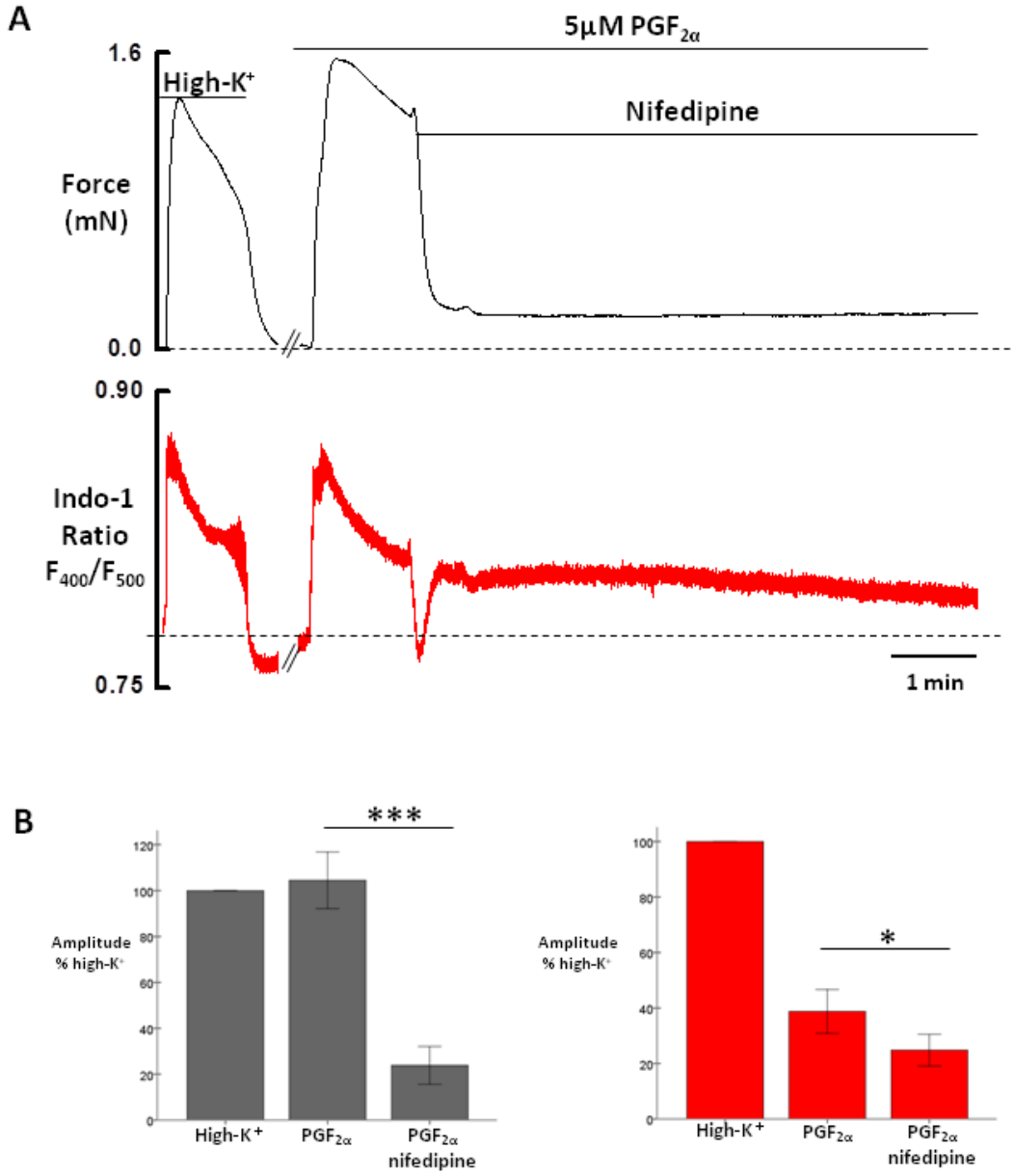

Figure 5.3.3.2 The effects of L-type VOCC inhibition on $PGF_{2\alpha}$ response in late gestation myometrial tissue. Inhibition of L-type VOCC (10 µM nifedipine) during the tonic component of 5 µM $PGF_{2\alpha}$ induced contraction resulted in a reduction of both force and $[Ca^{2+}]_i$, whilst also resulting in cessation of Ca^{2+} oscillations. (A) Original recordings of force (top trace) and $[Ca^{2+}]_i$ (bottom trace), initial drop in Ca^{2+} when nifedipine is added is due to movement artefact. (B) Graphs showing force (black) and Ca^{2+} (red), as % of peak initial high-K^+ for; high-K^+, $PGF_{2\alpha}$, and $PGF_{2\alpha}$ nifedipine. (n=5, *p<0.05, ***p<0.000)

5.3.4 The effects of removal and re-admission of external Ca^{2+} on pregnant rat myometrium

In order to investigate the possible role of SOCE, it is essential to establish the effects of removal and re-admission of external Ca^{2+} on uterine smooth muscle. Removal of external Ca^{2+} for both short term (5 minutes) and long term (15 minutes) in the presence of 2mM EGTA resulted in the cessation of Ca^{2+} transients and force production, whilst there was also a fall in basal $[Ca^{2+}]_i$. Upon re-admittance of Ca^{2+},$[Ca^{2+}]_i$ returned to normal levels and contractility profile returned to control levels (Figure 5.3.4). There was no overshoot of basal Ca^{2+} or force; basal $[Ca^{2+}]_i$ of 0.8577 ± 0.009 A.U. to 0.8477 ± 0.009 A.U. (*n*=5, p=0.06), while force went from 0.065 ± 0.6 mN to 0.053 ± 0.16 mN (*n*=5, p=0.686). This suggests that the SR was not depleted by removal of external Ca^{2+} or the mechanism of Ca^{2+} release Ca^{2+} entry coupling is not present in rat uterine smooth muscle.

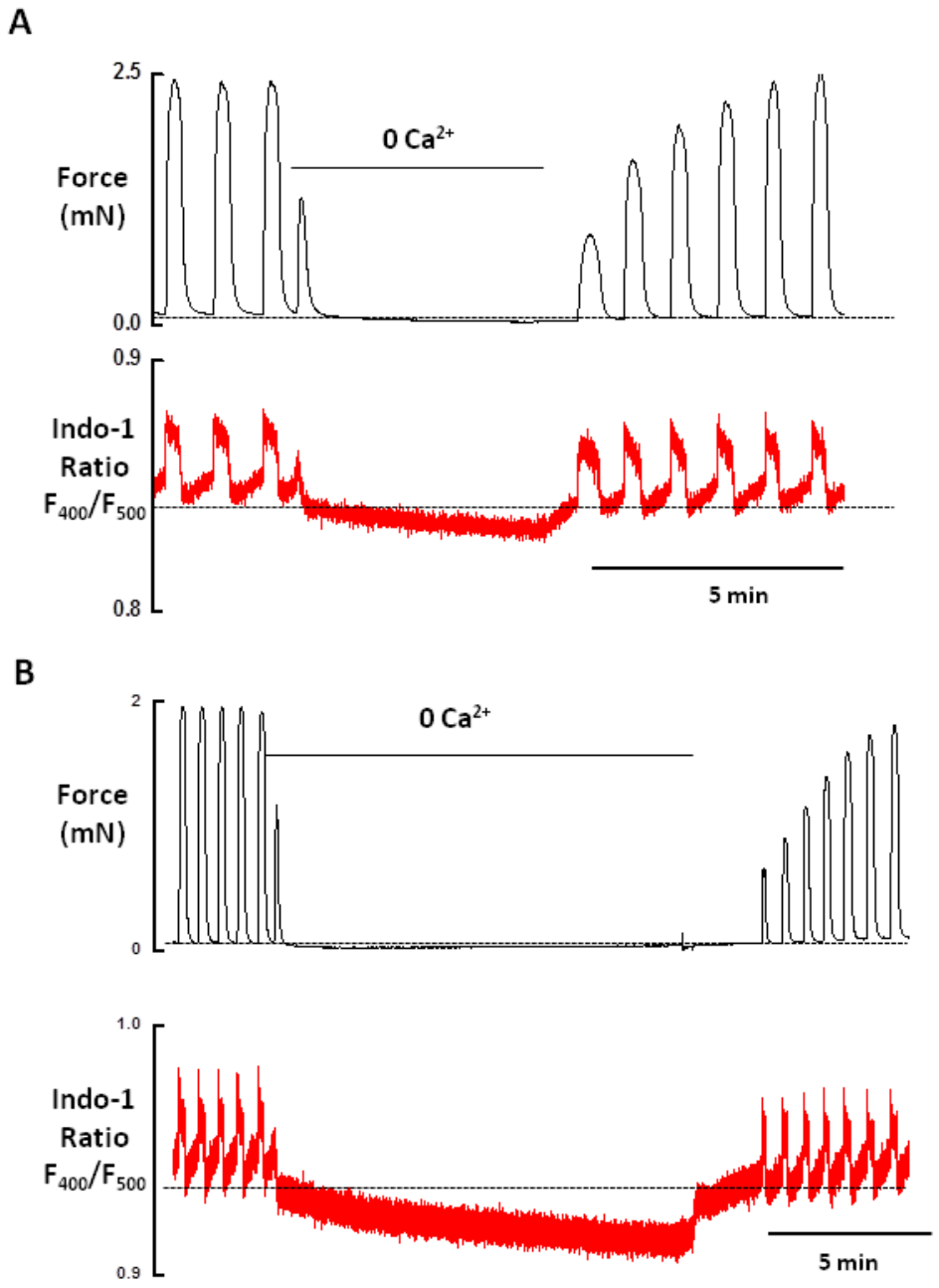

Figure 5.3.4 The effects of short and long term absence of external Ca^{2+} on contracting tissue in late gestation rat myometrium. Removal of external Ca^{2+} (2mM EGTA) results in a decrease in $[Ca^{2+}]_i$, addition of Ca^{2+} to the external bathing solution returns $[Ca^{2+}]_i$ to pre-treatment levels. Original records of force (top trace) and $[Ca^{2+}]_i$ (bottom trace) (A) 5 minutes (B) 15 minutes.

5.3.5 The effect of $PGF_{2\alpha}$ in the absence of external Ca^{2+}

To investigate the possibility that $PGF_{2\alpha}$ is able to activate SOC, it must be able to deplete the store. High-K^+ was first applied to the tissue to maximally fill the store and external Ca^{2+} removed in the presence of 2mM EGTA, $PGF_{2\alpha}$ in the continuing absence of external Ca^{2+} was applied in order to determine if agonists resulted in Ca^{2+} release from the store.

Using the photometric system with 50nM $F_{2\alpha}$, a concentration that increases phasic contractility, there was no detectable increase in either $[Ca^{2+}]_i$ or force production (*n*=5) (Figure 5.3.5.1(i)). However when 5µM $PGF_{2\alpha}$ was applied $[Ca^{2+}]_i$ increased by 34.87 ± 18.83 % of peak high-K^+ (*n*=6, p=0.002) and force increase by 8.12 ± 6.86 % (*n*=6, p=0.002) (Figure5.3.5.1(ii)). As with previous experiments this failure to detect changes in either force of $[Ca^{2+}]_i$ with 50nM $PGF_{2\alpha}$ may be due to lack of sensitivity of the photometric system. To verify this the same protocol was implemented using confocal microscopy to measure $[Ca^{2+}]_i$ in uterine strips. It can be clearly seen in figure 5.3.5.1(c) that 50nM $PGF_{2\alpha}$ in the absence of external Ca^{2+}, resulted in the oscillatory release of Ca^{2+} from the SR (*n* = 3), confirming that lack of changes in the photometric system are due to sensitivity levels, and so 5µM $PGF_{2\alpha}$ was used in further experiments to study store release and SOCE using the photometric system.

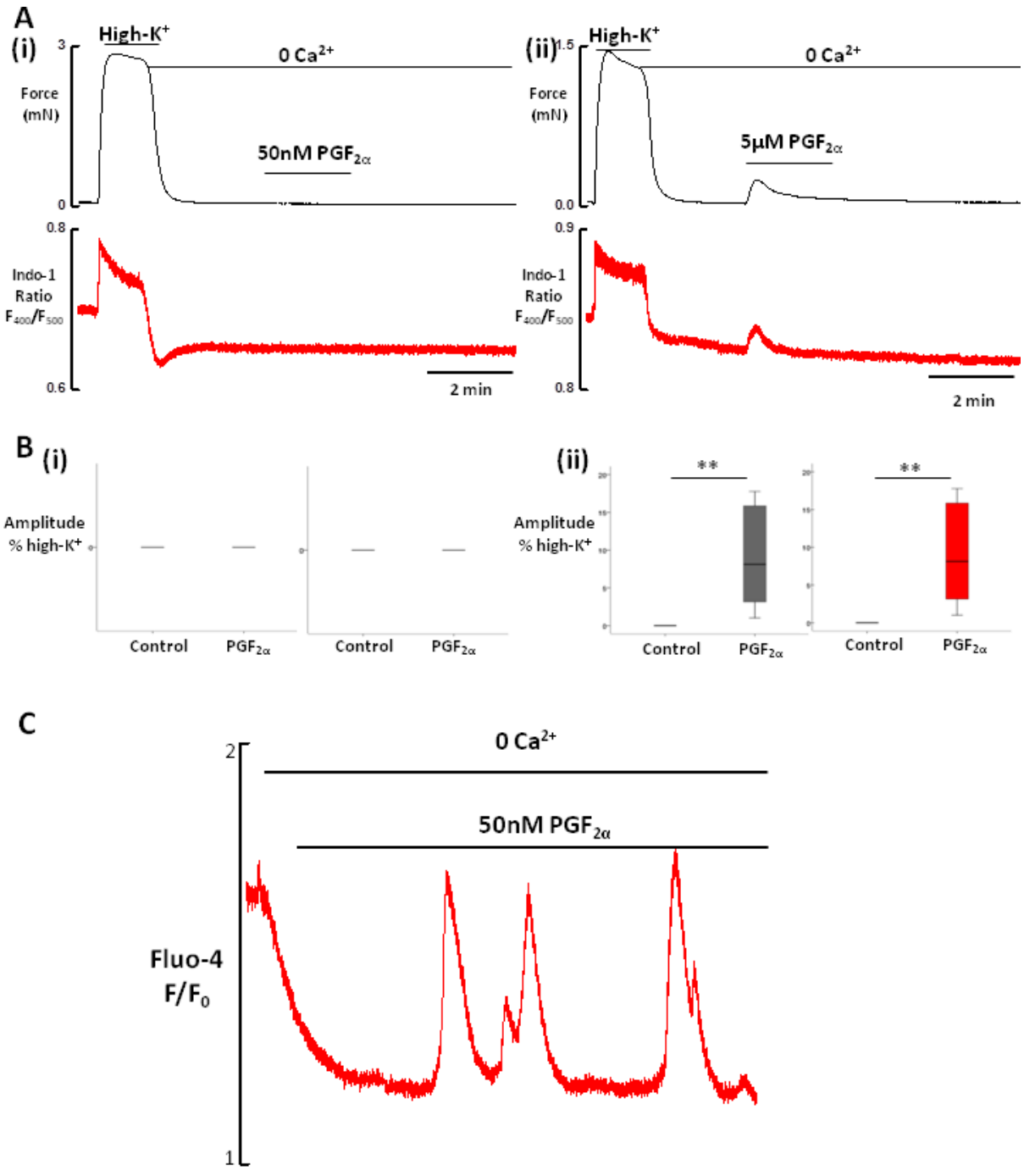

Figure 5.3.5.1 $PGF_{2\alpha}$ results in Ca^{2+} release from the SR, which can only been seen at higher concentrations on photometric system (A) Original records on photometric system showing response to application of (i) 50nM $PGF_{2\alpha}$ (n=5) (ii) 5μM $PGF_{2\alpha}$ (n=5) in the absence of external Ca^{2+}, force (top traces) and Ca^{2+} (bottom traces). (B) Graphs showing the increase in force and $[Ca^{2+}]_i$ in the absence of external Ca^{2+} compared to control (** $p<0.005$). (C) Confocal microscopy $[Ca^{2+}]_i$ recording of 50nM $PGF_{2\alpha}$ in the absence of external Ca^{2+} in uterine strips showing oscillatory Ca^{2+} releases (n=3).

Supramaximal concentration of $PGF_{2\alpha}$ resulted in release of Ca^{2+} from the store, increasing both $[Ca^{2+}]_i$ and force (Figure 5.3.5.2(i)). This increase in force and $[Ca^{2+}]_i$ is also seen with other myometrial agonists known to cause SR Ca^{2+} release; Carbachol (CCh) resulted in an increase in force of 27.97 ± 43.34 % of peak high-K^+ (*n*=5, p=0.003) and an increase in $[Ca^{2+}]_i$ of 38.59 ± 18.72 % (*n*=5, p=0.002) (Figure 5.3.5.2 (ii)), while adenosine tri-phosphate (ATP) increased force to 14.41 ± 9.18 % of peak high-K^+ (*n*=5, p=0.003), with an increase in $[Ca^{2+}]_i$ to 72.78 ± 13.76 % of peak high-K^+ (*n*=5, p=0.003) (Figure 5.3.5.2 (iii)).

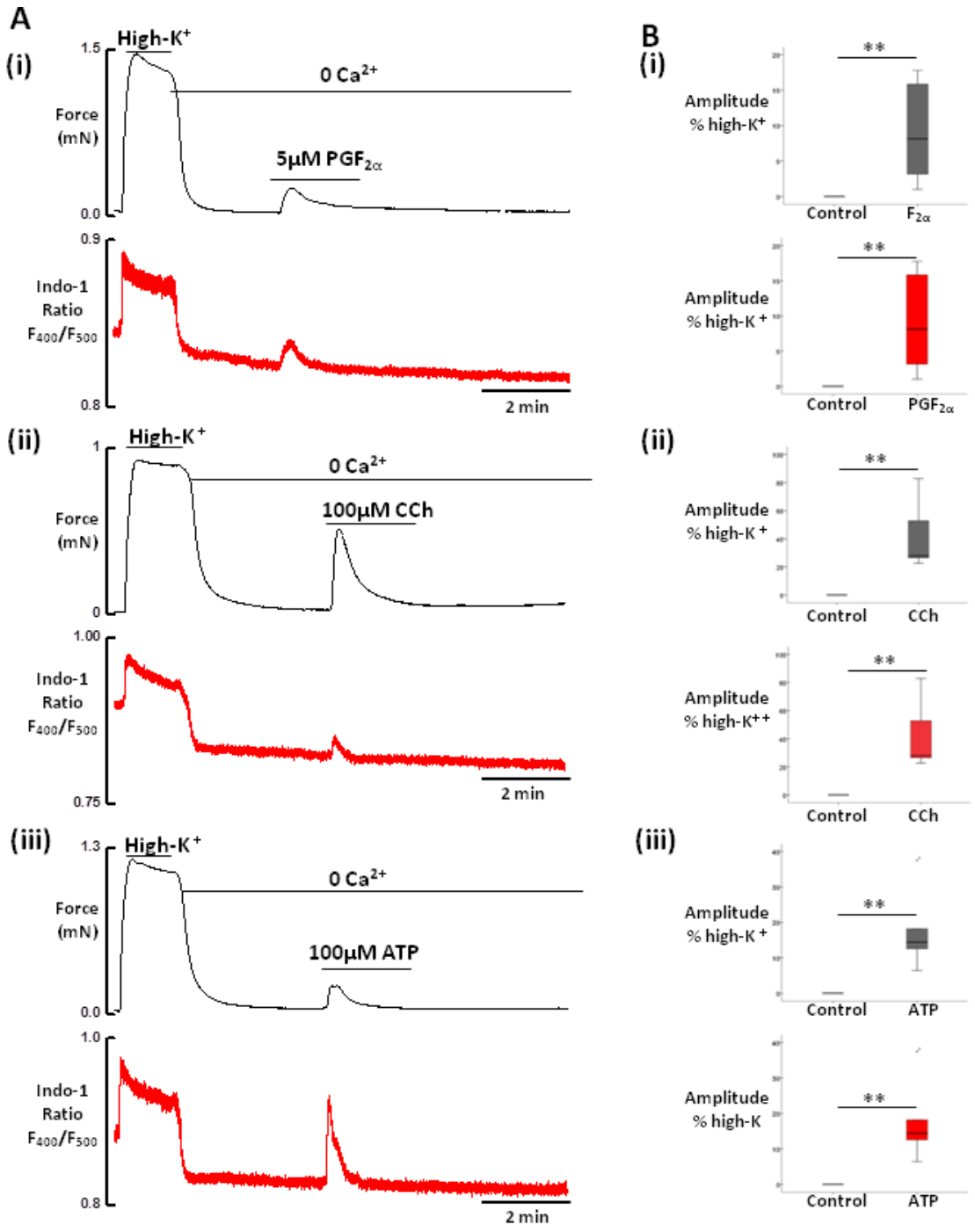

Figure 5.3.5.2 The effects of $PGF_{2\alpha}$ in the absence of external Ca^{2+} (A) Original records of force (top trace) and $[Ca^{2+}]_i$ (bottom trace), for; (i) $PGF_{2\alpha}$ (5 μM) (*n*=6) (ii) CCh (100 μM) (*n*=5) and (iii) ATP (100 μM) (*n*=5). Application of 5 μM $PGF_{2\alpha}$ and other agonists produced a transient rise in both force and $[Ca^{2+}]_i$ in the absence of external Ca^{2+}. (B) Graphs showing force (black) and Ca^{2+} (red), comparing agonist response to control. (** $p<0.005$)

To confirm Ca^{2+} release from the SR induced by $PGF_{2\alpha}$ and to study its temporal characteristics confocal microscopy of isolated cells myocytes in the absence of external Ca^{2+} was performed (*n* = 11 cells, 5 animals). $PGF_{2\alpha}$ resulted in the generation of Ca^{2+} waves, which were initiated at one end of the cell and propagating to the other (Figure 5.3.5.3b). Ca^{2+} waves had the following characteristics; time to peak 1.07 s, duration 3.67 s and time to relaxation 1.59 s and a speed of propagation of 87.23 μm/s (Figure 5.3.5.3a).

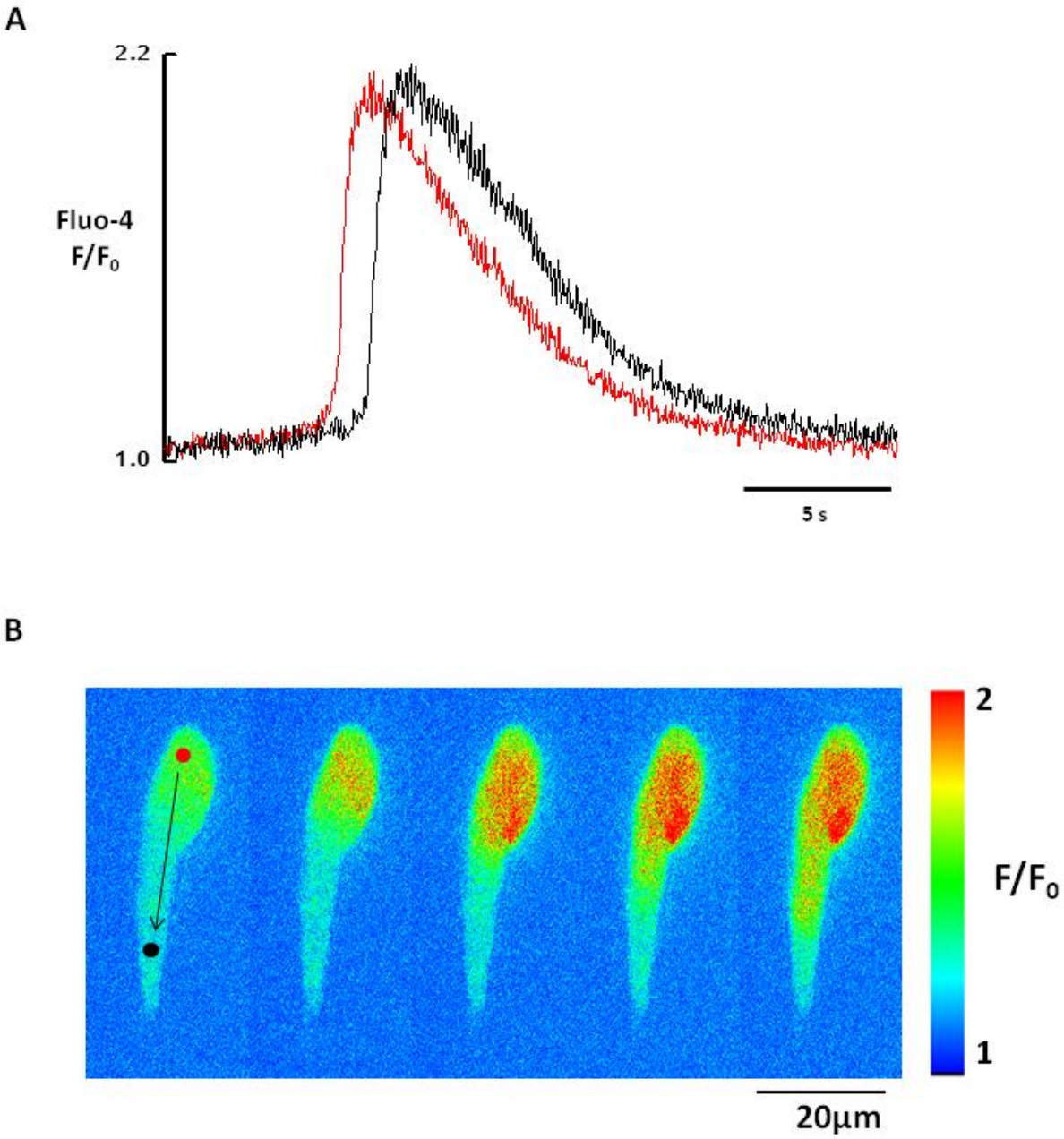

Figure 5.3.5.3 The effects of $PGF_{2\alpha}$ on Ca^{2+} characteristics in myometrial strips and isolated myocytes in the absence of external Ca^{2+}. Confocal microscopy of $PGF_{2\alpha}$ induced Ca^{2+} transients in the absence of external Ca^{2+}, black and red denote 2 regions of interest at either end of a cell, and arrow the direction of wave. (A) Original trace of Ca^{2+} wave in isolated myocyte (n= 11 cells, 5 animals) (B) Ca^{2+} wave induced by $PGF_{2\alpha}$ in an isolated myocyte at 200ms intervals.

5.3.6 The effects of L-type VOCC inhibition on sustained contraction induced by $PGF_{2\alpha}$ and CPA

$PGF_{2\alpha}$ causes Ca^{2+} release from the SR, but is it able to activate store-operated Ca^{2+} entry? A principal tool to investigate SOCE especially in smooth muscle is the SERCA inhibitor CPA, which has been previously used (Noble et al. 2009), and whose effects are not contaminated by ROC or downstream effects of PLC. In these and future experiments investigating SOCE, CPA is in the protocol or used as a comparison of the effects of $PGF_{2\alpha}$.

Previous evidence has linked SOCE to a sustained raise in $[Ca^{2+}]_i$ upon application of agonists. When L-type VOCC were inhibited (10µM nifedipine) during the sustained contraction of $PGF_{2\alpha}$, whilst there was a reduction in $[Ca^{2+}]_i$ and force, a significant proportion remained, immune to nifedipine. Basal force was maintained at 23.88 ± 4.12 % of peak high-K^+ (*n*=5, p=0.044), and $[Ca^{2+}]_i$ at 65.98 ± 3.46 % (*n*=5, p=0.001) (Figure 5.3.6(ii)). CPA like $PGF_{2\alpha}$ also resulted in a sustained nifedipine resistant increase in force and $[Ca^{2+}]_i$; force was maintained at 64.79 ± 12.18 % of peak high-K^+ (*n*=5, p=0.005) and $[Ca^{2+}]_i$ at 67.57 ± 10.58 % (*n*=5, p=0.005) (Figure 5.3.6 (i)).

The maintenance of nifedipine resistant $[Ca^{2+}]_i$ and force could be due to a number of factors, including; ROC, NSCC and SOCE. Comparison of response of $PGF_{2\alpha}$ to CPA, suggests that it is probable that this response is due to SOCE, although further work is needed to confirm this.

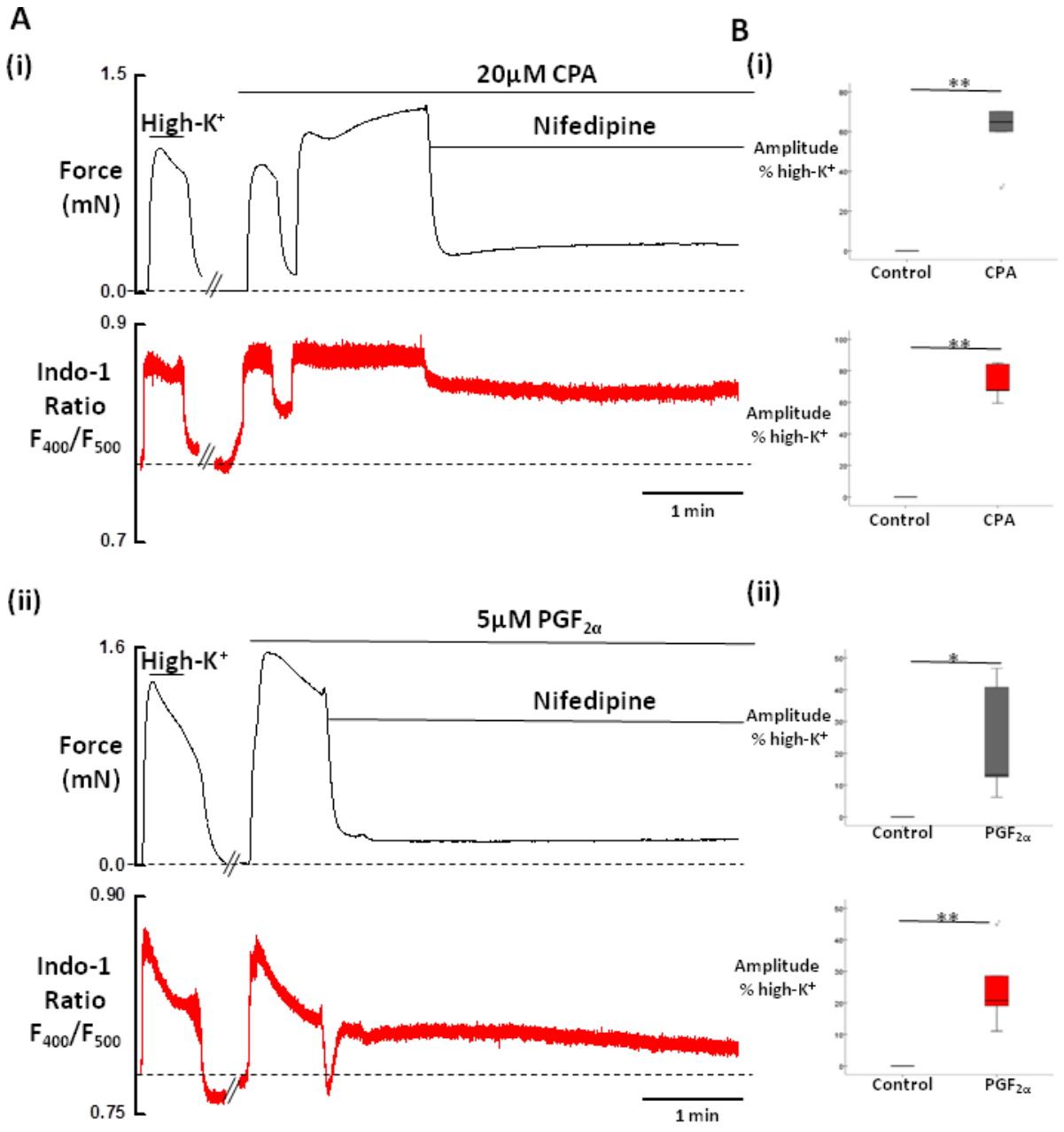

Figure 5.3.6 The effects of L-type VOCC inhibition on $PGF_{2\alpha}$ & CPA contracting tissue. Inhibition of L-type VOCC during the tonic contraction induced by (i) 20 µM CPA (ii) 5 µM $PGF_{2\alpha}$ results in a nifedipine resistant production of force and enhanced $[Ca^{2+}]_i$. (A) Original records of force (top traces) and $[Ca^{2+}]_i$ (bottom traces). (B) Graphs comparing agonist in the presence of nifedipine amplitude to control (no agonist); force (black) and Ca^{2+} (red). (n=5, * p<0.05, ** p<0.005)

5.3.7 The effects of re-admittance of external Ca^{2+} following $PGF_{2\alpha}$ and CPA induced store depletion

To investigate the rebound effects of store depletion by $PGF_{2\alpha}$ on re-admittance of Ca^{2+} to the external bathing solution, the store depletion protocol was extended. The SR was filled by high-K^+ stimulation, Ca^{2+} removed from the external bathing solution in the presence of 2mM EGTA, $PGF_{2\alpha}$ (5µM) or CPA (20µM) was added in the continuing absence of Ca^{2+} and following a short washout period Ca^{2+} was re-admitted to the external bathing solution.

Re-admittance of Ca^{2+} after store depletion induced by $PGF_{2\alpha}$ resulted in the initiation of spontaneous activity and an increase in both basal $[Ca^{2+}]_i$ and force. $[Ca^{2+}]_i$ increased from 37.73 ± 7.31 % of peak high-K^+ in control experiments to 91.58 ± 13.78 % (*n*=6, p=0.007), while force also increased from 2.37 ± 0.72 % of peak high-K^+ to 16.33 ± 2.80 % (*n*=6, p=0.003) (Figure 5.3.7.1).

A similar response was seen when the store was depleted with CPA, upon re-admittance of Ca^{2+} spontaneous activity was initiated. Spontaneous activity which was phasic in all $PGF_{2\alpha}$ samples, was only phasic in 2 out of the 6 samples tested with CPA, in the remaining 4 samples response was tonic-like, until it returned to basal levels. As with $PGF_{2\alpha}$ there was an enhanced basal $[Ca^{2+}]_i$ and force. $[Ca^{2+}]_i$ increased from 37.73 ± 7.77 % of peak high-K^+ to 74.25 ± 10.25 % (*n*=5, p=0.0018), while force increased from 2.37 ± 0.74 % of peak high-K^+ to 59.33 ± 8.87 % (*n*=5, p=0.001) (Figure 5.3.7.2).

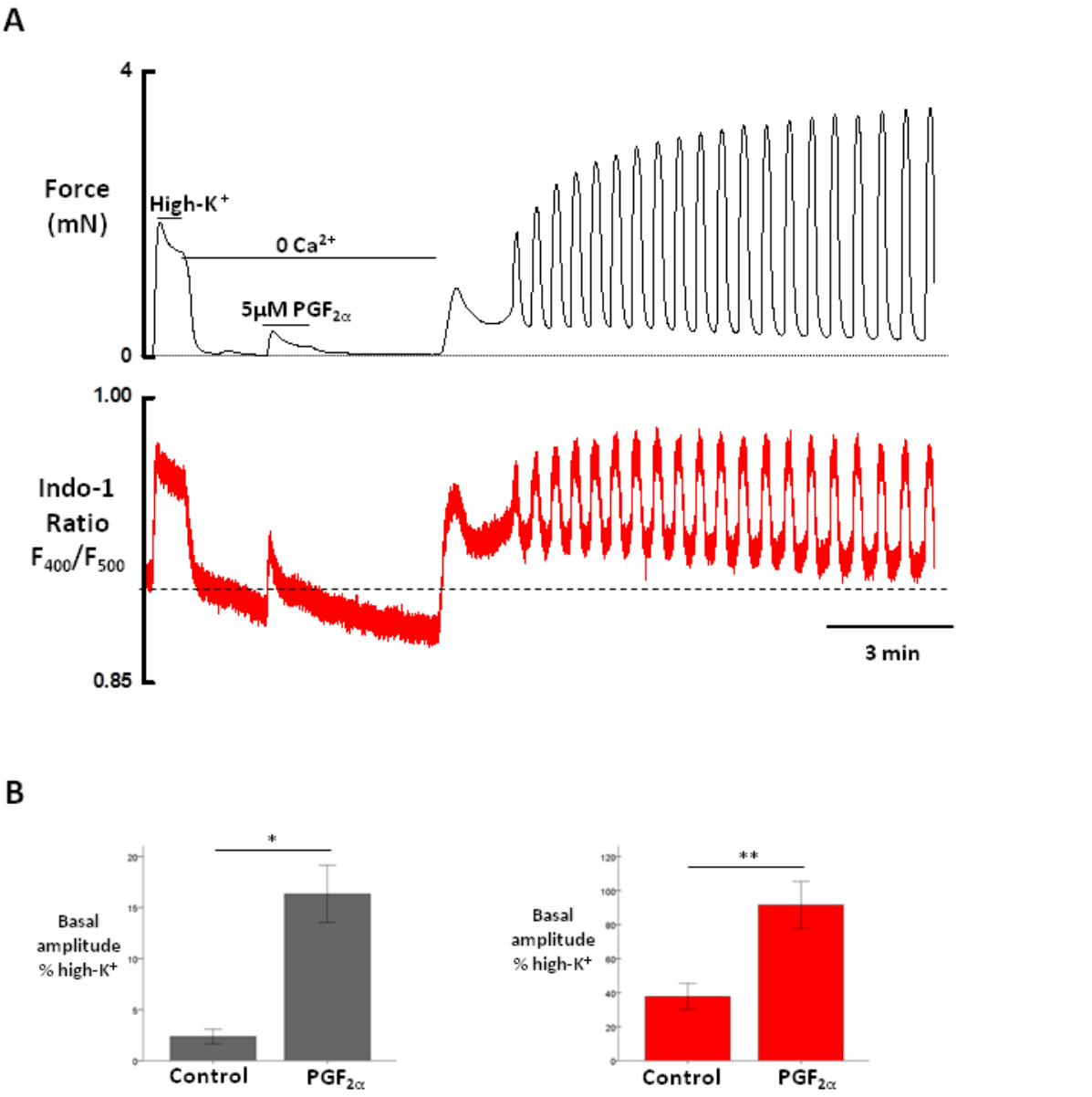

Figure 5.3.7.1 The effects of $PGF_{2\alpha}$ induced store depletion (A) Original record of force (top trace) and $[Ca^{2+}]_i$ (bottom trace) showing store depletion induced by 5 µM $PGF_{2\alpha}$ in the absence of external Ca^{2+}, and rebound effects on re-admittance of Ca^{2+}.(B) Graphs showing enhanced basal force (black) and $[Ca^{2+}]_i$ (red) upon re-admittance of Ca^{2+} compared to control. (n=6, * p<0.05, ** p<0.005).

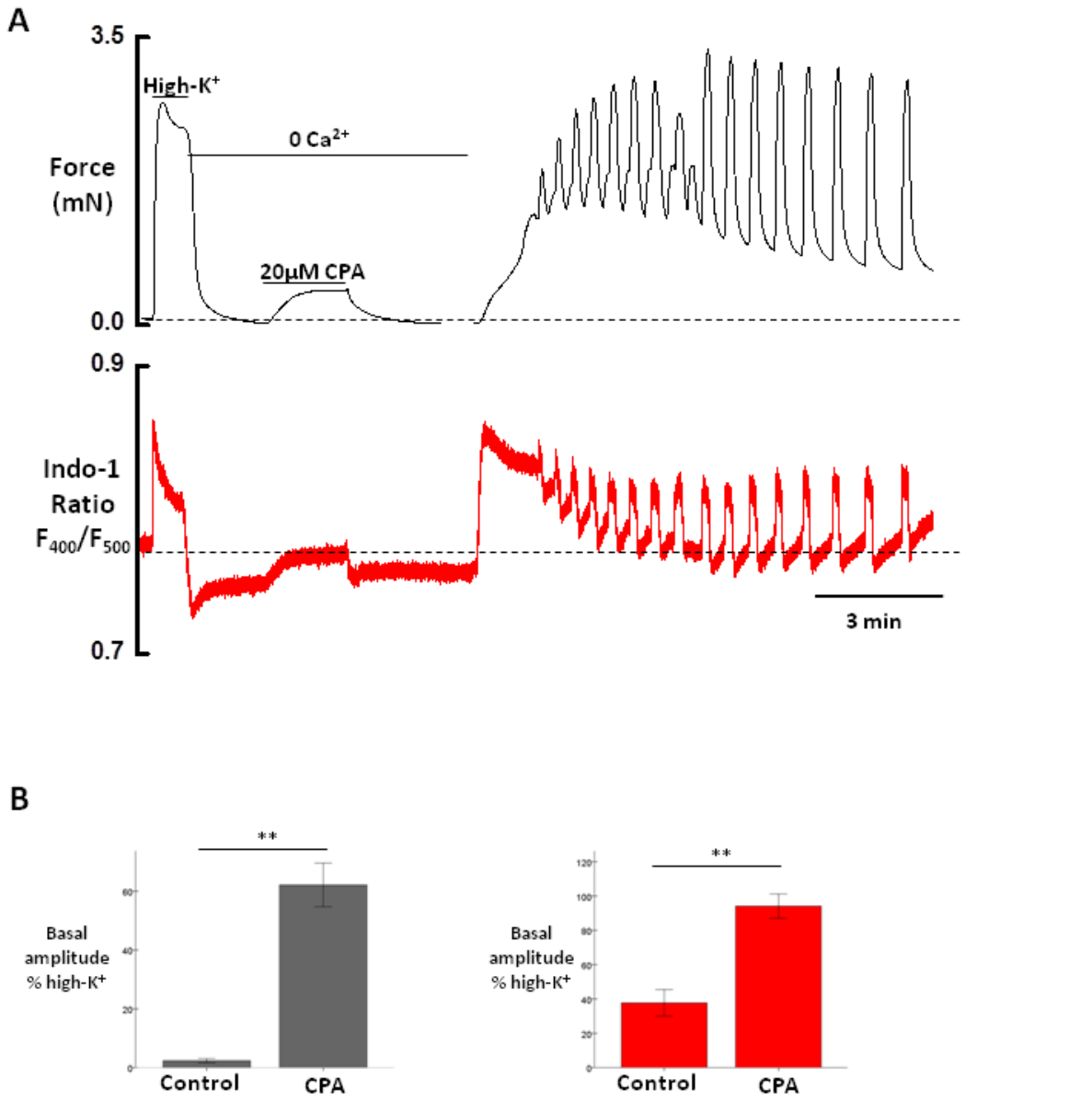

Figure 5.3.7.2 The effects of CPA induced store depletion. (A) Original record of force (top trace) and $[Ca^{2+}]_i$ (bottom trace) showing store depletion induced by 20 μM CPA in the absence of external Ca^{2+}, and rebound effects upon re-admittance of Ca^{2+}. (B) Graphs showing enhanced basal force (black) and $[Ca^{2+}]_i$ (red) upon re-admittance of Ca^{2+} compared to control. (n=6, ** $p<0.005$)

5.3.8 The effects of L-type VOCC inhibition on rebound effect induced by $PGF_{2\alpha}$ and CPA store depletion

The enhanced rebound effect seen upon re-admittance of Ca^{2+} after store depletion by CPA can be due to a number of mechanisms; firstly by L-type Ca^{2+} channels, ROC/NSCC or by SOCE. To exclude L-type VOCC in the rebound effect of CPA and $PGF_{2\alpha}$, the L-type VOCC inhibitor, nifedipine (10μM) was added during the rebound phase.

Inhibition of L-type VOCC had no effect on the rebound basal force or $[Ca^{2+}]_i$ induced by $PGF_{2\alpha}$. Basal force in control experiments was 17.09 ± 4.47 % of peak high-K^+ compared to 30.99 ± 7.77 % when L-type VOCC were inhibited (*n*=5, p=0.159), $[Ca^{2+}]_i$ was 59.19 ± 55.22 % of peak high-K^+ and 80.64 ± 57.63 % (*n*=5, p=0.078) (Figure 5.3.8.1). This was also the case for CPA induced rebound effects, with basal force remaining the same from 47.20 ± 6.55 % of peak high-K^+ to 52.96 ± 11.98 % (*n*=5, p=0.685), while $[Ca^{2+}]_i$ increased from 65.39 ± 6.91 % of peak high-K^+ to 111.24 ± 4.33 % (*n*=5, p=0.023) (Figure 5.3.8.2). The increase in $[Ca^{2+}]_i$ with the addition of nifedipine onto CPA contracting tissue, is likely due to a slight change in temperature, to which rat uterine tissue is particularly sensitive and / or slight movement artifact during nifedipine treatment due to constraints within the methodology. In $PGF_{2\alpha}$ experiemtns there was a trend increase in $[Ca^{2+}]_i$ although this did not reach significance.

L-type VOCC do not play a role in the enhanced basal force and $[Ca^{2+}]_i$ seen with re-admittance of Ca^{2+} after store depletion, although they are pivotal for the phasic activity seen. As CPA has similar response to that seen with $PGF_{2\alpha}$, which cannot be due to ROC/NSCC, this strongly suggests that the activation of Ca^{2+} entry following Ca^{2+} depletion of the store by both $PGF_{2\alpha}$ and CPA is due to activation of SOCE.

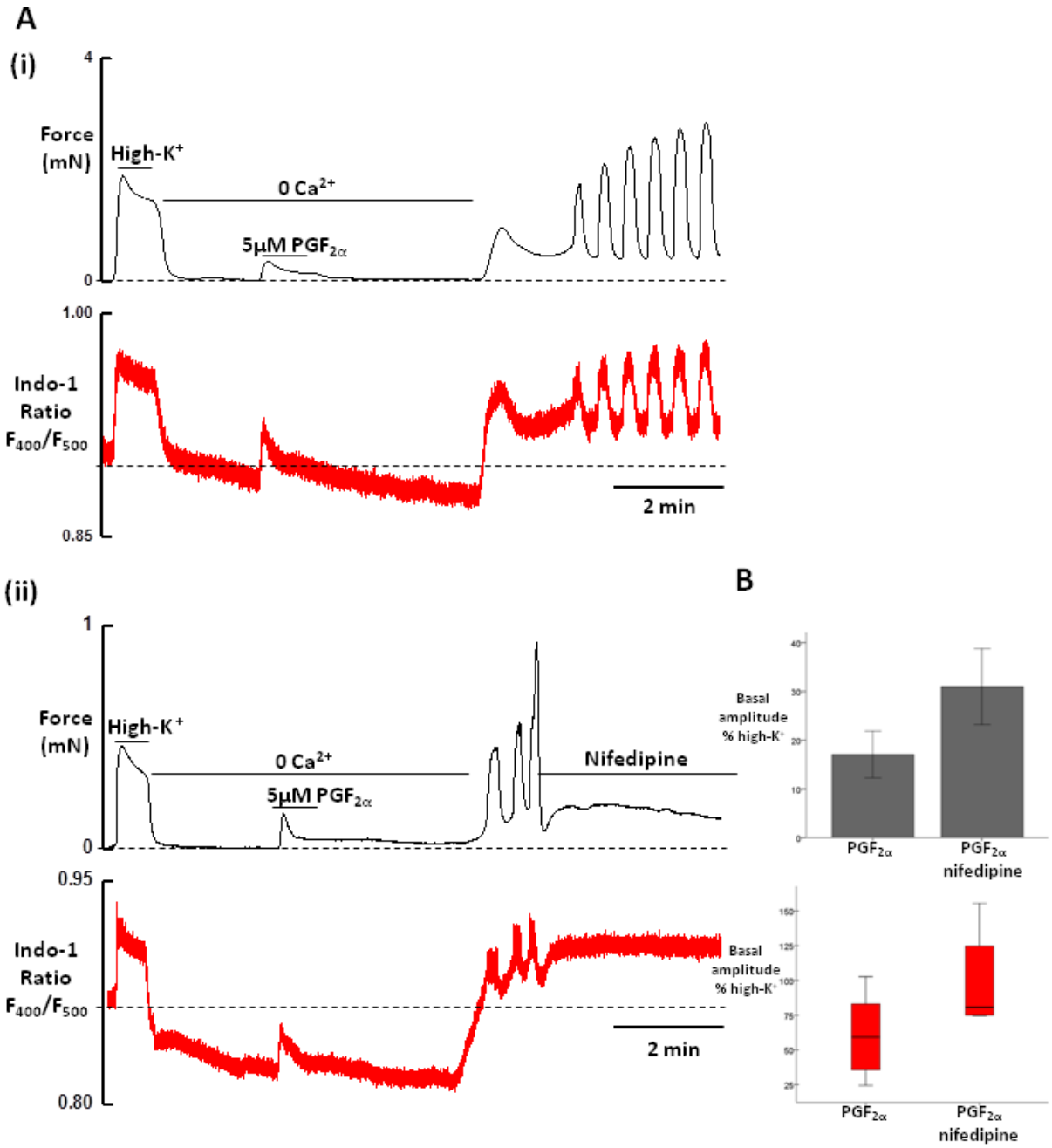

Figure 5.3.8.1 The effects of L-type VOCC inhibition on rebound basal force and $[Ca^{2+}]_i$ induced by $PGF_{2\alpha}$ store depletion. (A) Original records of force (top traces) and $[Ca^{2+}]_i$ (bottom traces) showing abolishment of Ca^{2+} transients and oscillations in force with inhibition of L-type VOCC (10 µM nifedipine) (i) control, (ii) nifedipine. (B) Graphs showing no change in enhanced basal force (black) or $[Ca^{2+}]_i$ (red) on L-type VOCC inhibition. (n=5)

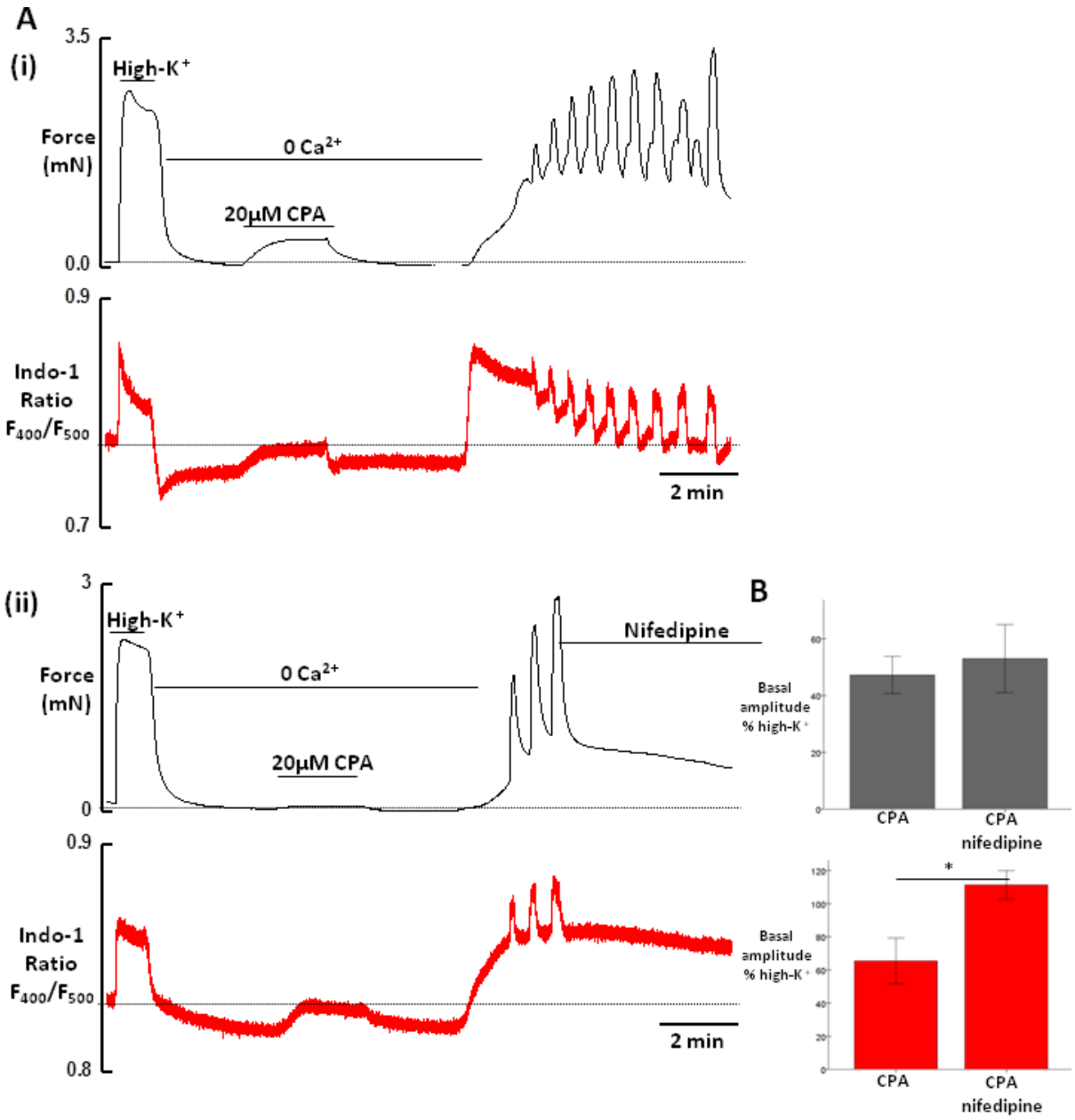

Figure 5.3.8.2 The effects of L-type VOCC inhibition on rebound basal force and $[Ca^{2+}]_i$ induced by CPA store depletion. (A) Original records of force (top traces) and $[Ca^{2+}]_i$ (bottom traces) showing abolishment of Ca^{2+} transients and oscillations in force with inhibition of L-type VOCC (10 µM nifedipine) (i) control, (ii) nifedipine. (B) Graphs showing no change in enhanced basal force (black) or $[Ca^{2+}]_i$ (red) on L-type VOCC inhibition. (n=6)

5.3.9 The effects of the SOCE inhibitors Gd^{3+} and La^{3+} on CPA rebound

To confirm that the enhanced rebound effects seen with CPA in myometrial tissue is due to SOCE, the previous store depletion protocol was used in the presence of either La^{3+} or Gd^{3+}; two non-specific inhibitors of SOCE. Both have been used extensively to study SOCE (Miyoshi et al. 2004; Noble et al. 2009).

In the presence of La^{3+} basal force was substantially reduced, falling from 59.33 ± 8.87 % of peak high-K^+ to 5.99 ± 2.63 % (*n* = 5, p = 0.001), which was not reflected in $[Ca^{2+}]_i$, from 64.33 ± 16.95 % of peak high-K^+ to 33.57 ± 27.56 % (*n*=5, p=0.890) (Figure 5.3.9(ii)). This was replicated with Gd^{3+}; basal force falling from 59.33 ±8.87 % of peak high-K^+ to 12.67 ± 4.03 % (*n*=5, p=0.007), which was also not reflected in $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ remaining unchanged from 74.25 ± 10.25 % if peak high-K^+ to 68.06 ± 5.92 % (*n*=5, p=0.633) (Figure 5.3.9(iii)). In addition to the reduction in basal force, with the use of either inhibitor, the phasic contractility and concomitant Ca^{2+} transients seen in 2 of the 6 control strips was abolished.

These experiments in part suggest that CPA works through SOCE in pregnant rat myometrium, in consensus with previously published data (Yang et al. 2002; Shlykov et al. 2003; Noble et al. 2009). While SOCE was demonstrated by the reduction in enhanced basal force induced by the two inhibitors, it was not reflected in intracellular Ca^{2+} measurements. This lack of Ca^{2+} reduction in the presence of La^{3+} and Gd^{3+} is probably due to an inherent problem with the photometric measurement of $[Ca^{2+}]_i$. Large areas of tissue are measured in the photometric system and if tissue is not well synchronised, can lead to problems such as small Ca^{2+} transients, increasing levels of variation, this along with lack of sensitivity can result in erroneous non-significant results. Additionally both La^{3+} and Gd^{3+} are likely to affect Indo-1 fluorescence which could be one of the experimental pitfalls of using this indicator.

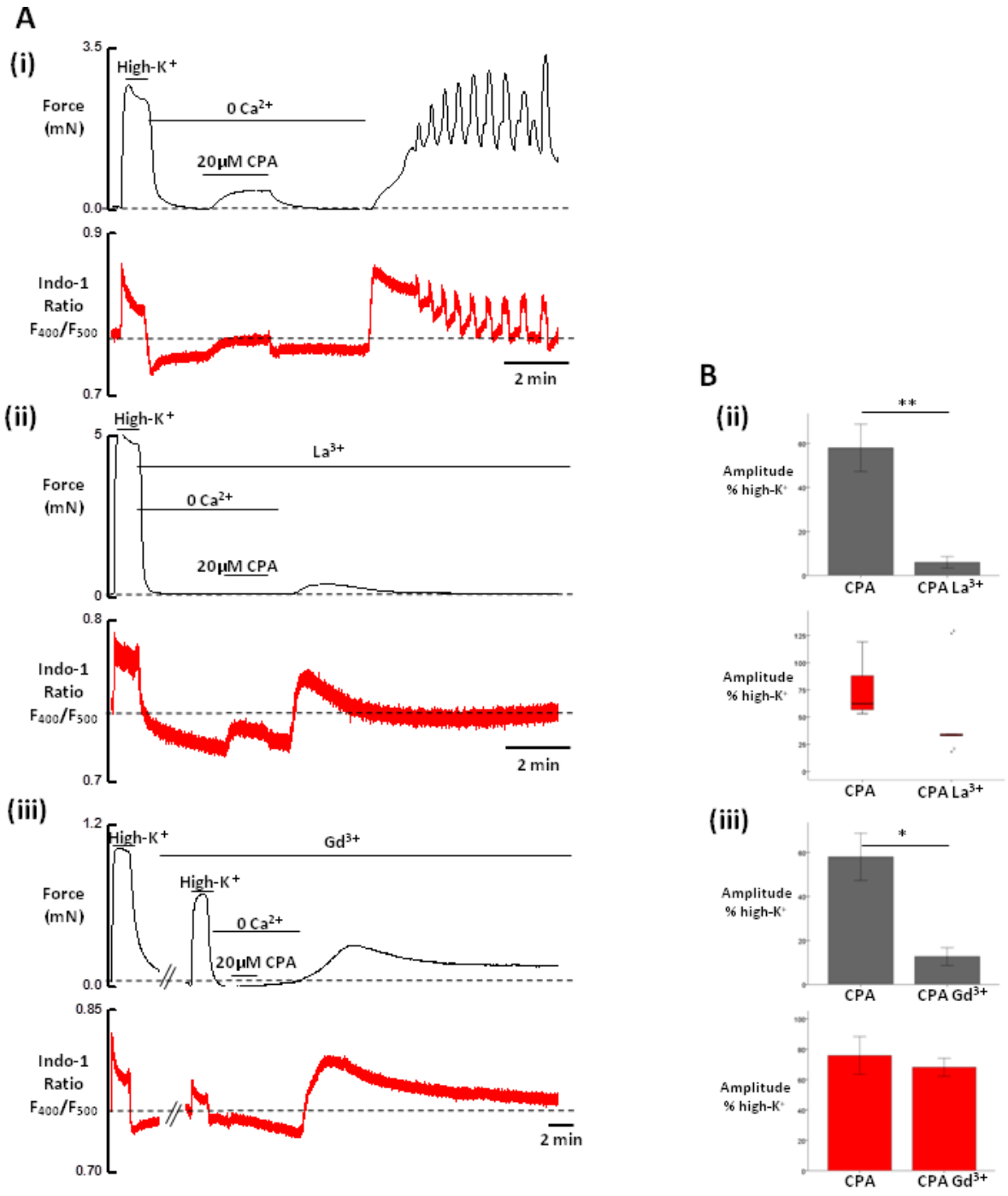

Figure 5.3.9 SOCE inhibition reduces enhanced rebound effects induced by CPA store depletion. (A) Original records of force (top traces) and $[Ca^{2+}]_i$ (bottom traces) showing abolishment of enhanced basal force and $[Ca^{2+}]_i$, with (ii) La^{3+} (10 µM), (iii) Gd^{3+} (10 µM), compared to (i) control. (B) Graphs showing effects of SOCE inhibition; force (black), and Ca^{2+} (red). (n=5, * p<0.05, ** p<.005)

5.3.10 The effects of the SOCE inhibitors Gd^{3+} and La^{3+} on $PGF_{2\alpha}$ rebound

In order to determine if SOCE plays a part in the enhanced rebound effects seen with $PGF_{2\alpha}$, CPA SOCE inhibitor experiment was repeated in the presence of $PGF_{2\alpha}$.

Similar to the effects of La^{3+} on CPA, both rebound force and $[Ca^{2+}]_i$ induced by $PGF_{2\alpha}$ was greatly reduced in the presence of the SOCE inhibitor, La^{3+} (Figure 5.3.10(ii)). Basal force fell from 16.33 ± 2.80 % of peak high- K^+ to 5.10 ± 1.78 % (*n*=6, p=0.007), while $[Ca^{2+}]_i$ also reflected this, falling from 91.58 ± 33.76 % of peak high-K^+ to 39.17 ± 6.09 % (*n*=6, p=0.006). Unlike CPA rebound, La^{3+} did not inhibit phasic contractility induced by re-admission of Ca^{2+}, although the length of contractility was significantly reduced.

Unlike La^{3+}, Gd^{3+} did not affect basal force of rebound induced by $PGF_{2\alpha}$ store depletion. Basal force in control conditions was 16.33 ± 2.80 % of peak high-K^+ compared to 22.31 ± 7.13 % (*n*=5, p=0.479) in the presence of Gd^{3+}. Although there was a reduction in basal $[Ca^{2+}]_i$, from 91.58 ± 13.78 % of peak high-K^+ in control conditions falling to 44.57 ± 8.50 % in the presence of Gd^{3+} (*n*=5, p=0.022) (Figure 5.3.10(iii)).

From these results it is probable that $PGF_{2\alpha}$ works to some extent through SOCE, La^{3+} did result in a reduction in both basal force and $[Ca^{2+}]_i$ although Gd^{3+} did not reflect this reduction in force. This discrepancy could be due to a number of factors; firstly channels involved in SOCE are not equally sensitive to all inhibitors of SOCE, especially those of the TRP family (Jung et al. 2003; Beech 2005; Helli et al. 2005; McElroy et al. 2008). With some smooth muscles possessing Gd^{3+} insensitive SOCE (Wilson et al. 2002). To date store operated calcium channels have not been identified in pregnant rat myometrium, so while both inhibitors block rebound force for CPA, CPA and $PGF_{2\alpha}$ may induced different subtype

of store operated Ca^{2+} channels. Secondly based on previous evidence (Coleman et al. 1994) and washout experiments (Chapter 3) $PGF_{2\alpha}$ has low dissociation constant, and so this experiment will to a degree be contaminated with ROC/NSCC and the downstream effects.

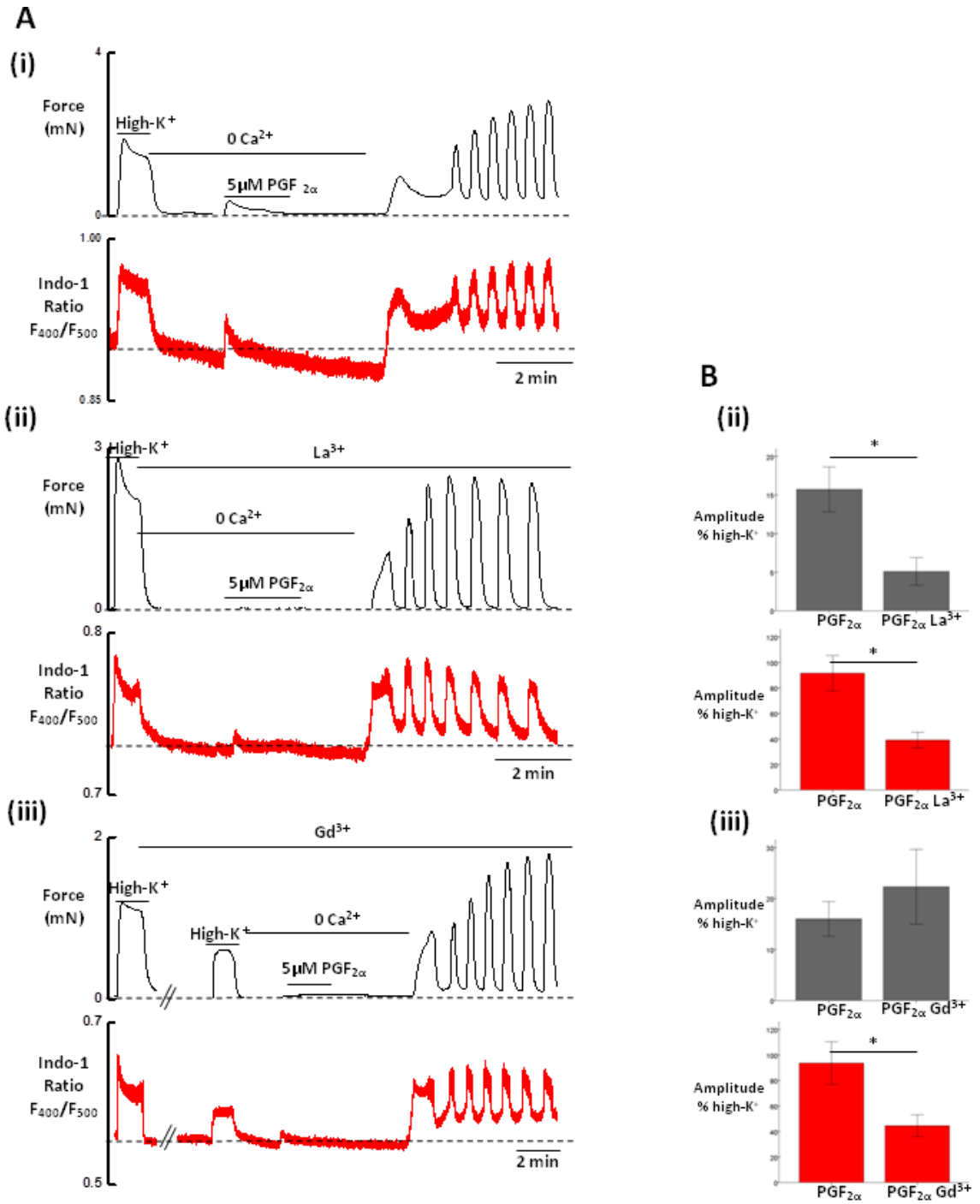

Figure 5.3.10 SOCE inhibition reduces enhanced rebound effects induced by $PGF_{2\alpha}$ store depletion. (A) Original records of force (top trace) and $[Ca^{2+}]_i$ (bottom trace) showing abolishment of enhanced basal force and $[Ca^{2+}]_i$, with (ii) La^{3+} (10 µM) ($n = 6$), and not with (iii) Gd^{3+} (10 µM) ($n = 5$), compared to (i) control. (B) Graphs showing effects of SOCE inhibition; force (black), and Ca^{2+} (red). (* $p<0.05$)

5.3.11 The effects of $PGF_{2\alpha}$ in the presence of CPA

There are four possible Ca^{2+} entry mechanisms in uterine smooth muscle; L-type VOCC, SOCE, ROC and NSCC. Using the SERCA inhibitor CPA (20µM), the SR is depleted and the membrane is depolarised leading to the initiation of spike discharge followed by its depolarisation block (Figure 5.3.11.1) (Burdyga, T. Unpublished data). Addition of 5µM $PGF_{2\alpha}$ in the presence of CPA resulted in an increase in both force and $[Ca^{2+}]_i$; force increased from 127.83 ± 6.56 % of peak high-K^+ to 151.29 ± 6.94 % (*n*=5, p=0.016), and $[Ca^{2+}]_i$ from 40.52 ± 35.57 % of peak high-K^+ to 52.51 ± 41.90 % (*n*=5, p=0.046) (Figure 5.3.11.2).

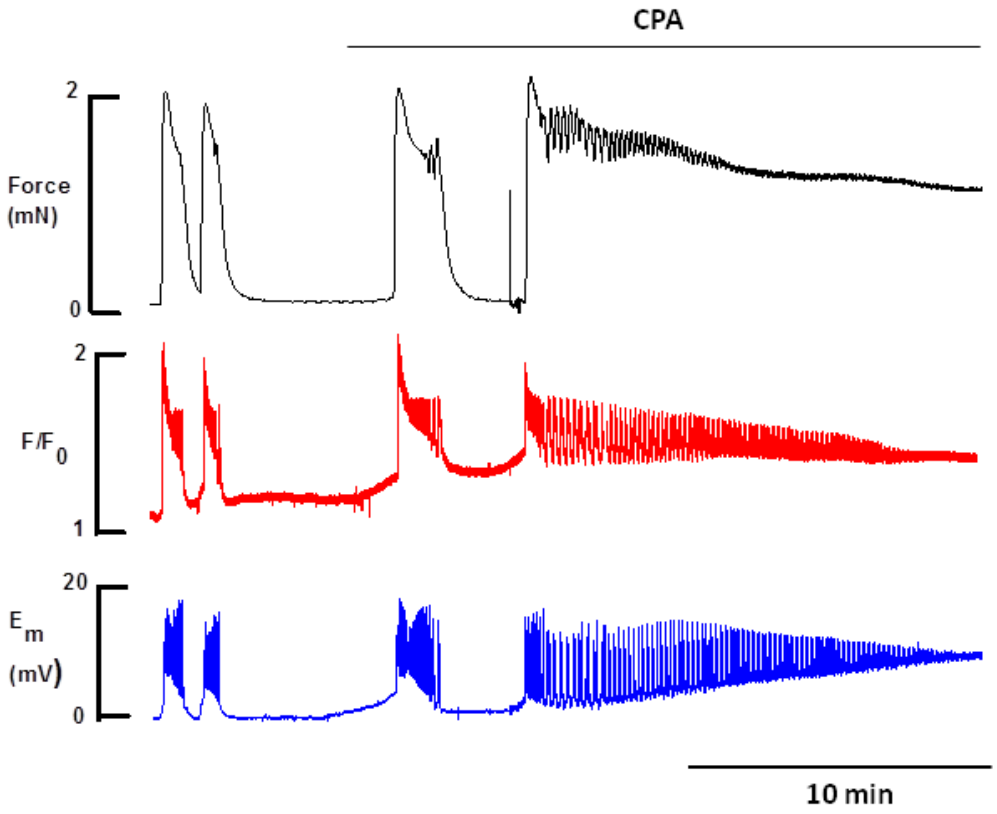

Figure 5.3.11.1 The effects of CPA on electrical activity, $[Ca^{2+}]_i$ and force. Original trace of electrical activity (blue), Ca^{2+} transient (red) and force (black) induced by CPA. Showing depolarisation of tissue, cessation of Ca^{2+} spikes and steady state force production (Burdyga T. unpublished data)

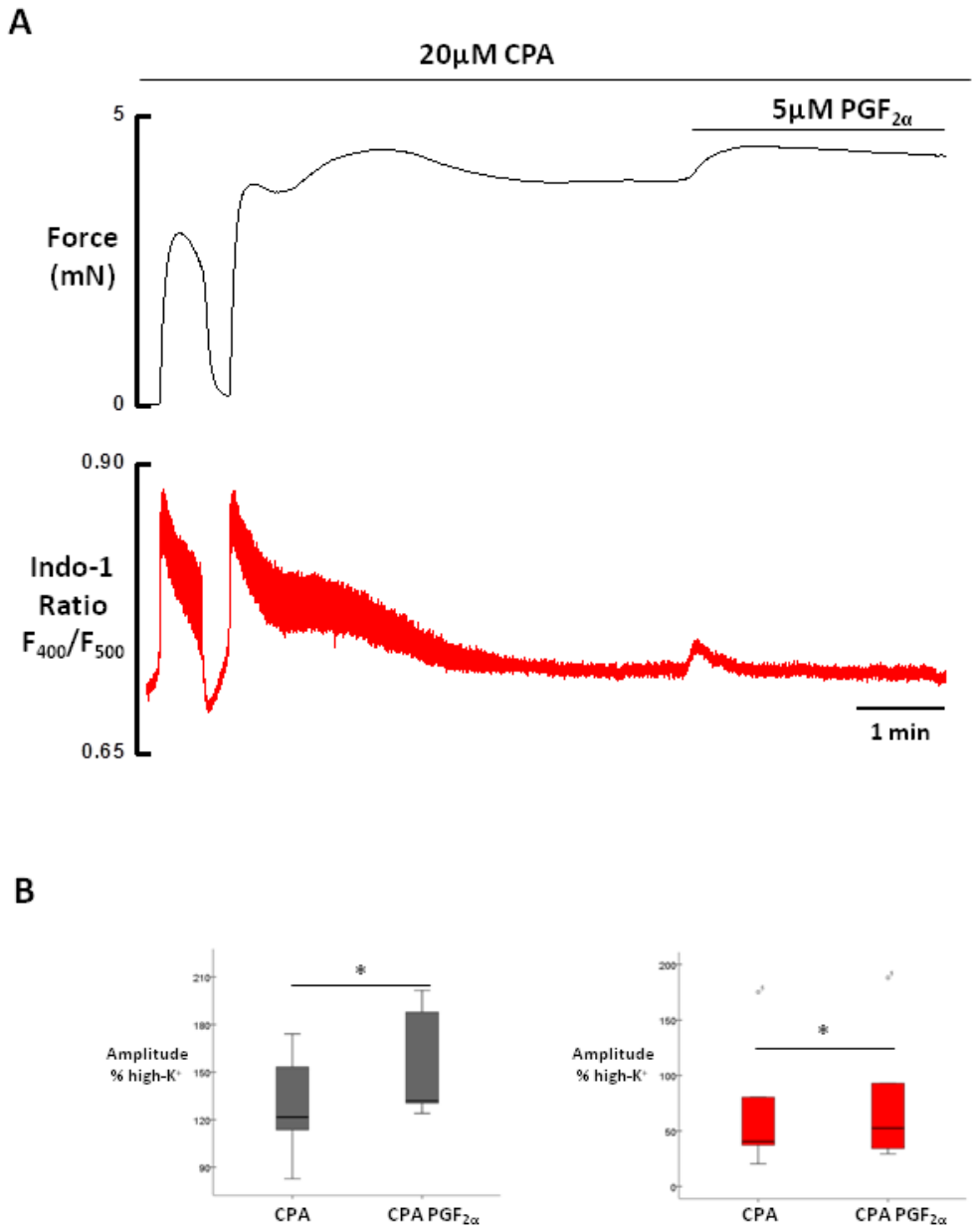

Figure 5.3.11.2 The effects of $PGF_{2\alpha}$ on CPA induced contracting tissue. (A) Original record of force (top trace) and $[Ca^{2+}]_i$ (bottom trace) showing the production of both force and an increase in $[Ca^{2+}]_i$ with the addition of 5 µM $PGF_{2\alpha}$ during the tonic contraction induced with 20 µM CPA.(B) Graphs showing the effects of $PGF_{2\alpha}$: force (black), and Ca^{2+} (red). (n=5, ** $p<0.05$)

Whilst CPA will result in opening of L-type VOCC the addition of $PGF_{2\alpha}$ may modulate the open probability, and so to ensure that L-type VOCC are not contributing to the increased force and $[Ca^{2+}]_i$ seen with the addition of $PGF_{2\alpha}$, the L-type VOCC inhibitor nifedipine was applied in the presence of CPA. $PGF_{2\alpha}$ resulted in an increase in force, from 47.08 ± 27.64 % of peak high-K^+ to 65.27 ± 27.55 % (*n*=6, p=0.046), whilst there was no significant change in $[Ca^{2+}]_i$; from 92.93 ± 13.14 % of peak high-K^+ to 98.13 ± 15.49 % (*n*=6, p=0.349) (Figure 5.3.11.3).

Removal of external Ca^{2+} produced a quick relaxation of both $[Ca^{2+}]_i$ and force induced by $PGF_{2\alpha}$ applied in the presence of combined application of CPA and nifedipine (Figure 5.3.11.4). Force returning to an amplitude of 5.28 ± 1.73 % peak of high-K^+, compared to pre-treatment levels of 3.24 ± 1.03 % (*n*=6, p=0.278).

The fact that $PGF_{2\alpha}$ can induce an increase in both $[Ca^{2+}]_i$ and force in the presence of CPA and an increase in force with the additional inhibition of L-type VOCC, lends support to the hypothesis that $PGF_{2\alpha}$ works through ROC and NSCC. Although the lack of an increase in $[Ca^{2+}]_i$ when L-type VOCC were inhibited suggests a failure in the sensitivity of the photometric system. NSCC play an important role in agonist response of smooth muscles, often with a Na^+ current, further work is needed to establish both the role of NSCC and the currents involved.

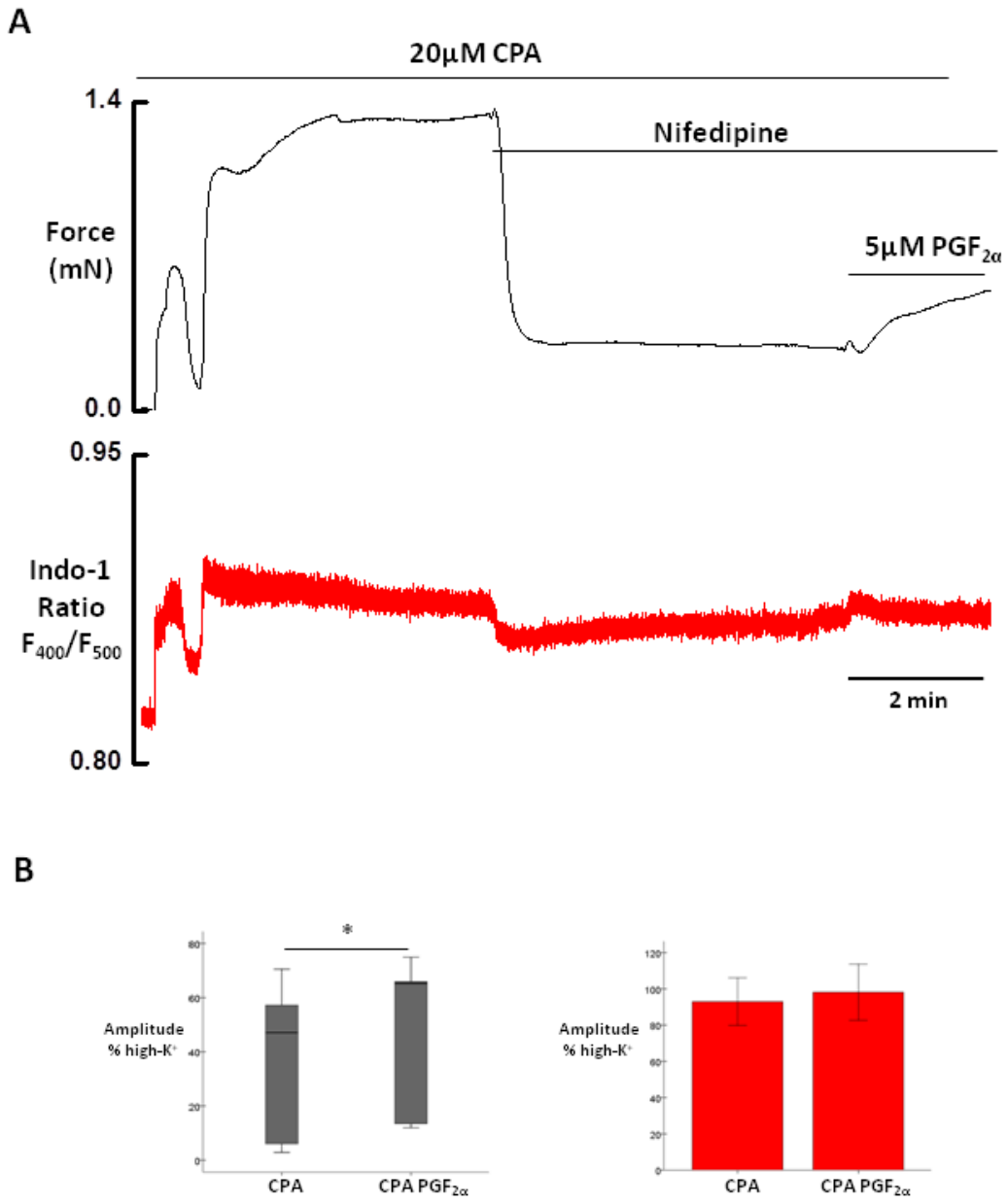

Figure 5.3.11.3 The effects of $PGF_{2\alpha}$ on CPA induced contracting tissue with the inhibition of L-type VOCC. (A) Original record of force (top trace) and $[Ca^{2+}]_i$ (bottom trace) showing the production of both force and an increase in $[Ca^{2+}]_i$ with the addition of 5 µM $PGF_{2\alpha}$ onto the tonic contraction induced with 20 µM CPA whilst L-type VOCC are inhibited (10 µM nifedipine). (B) Graphs showing effects of $PGF_{2\alpha}$: force (black), and Ca^{2+} (red). (n=6, * $p<0.05$)

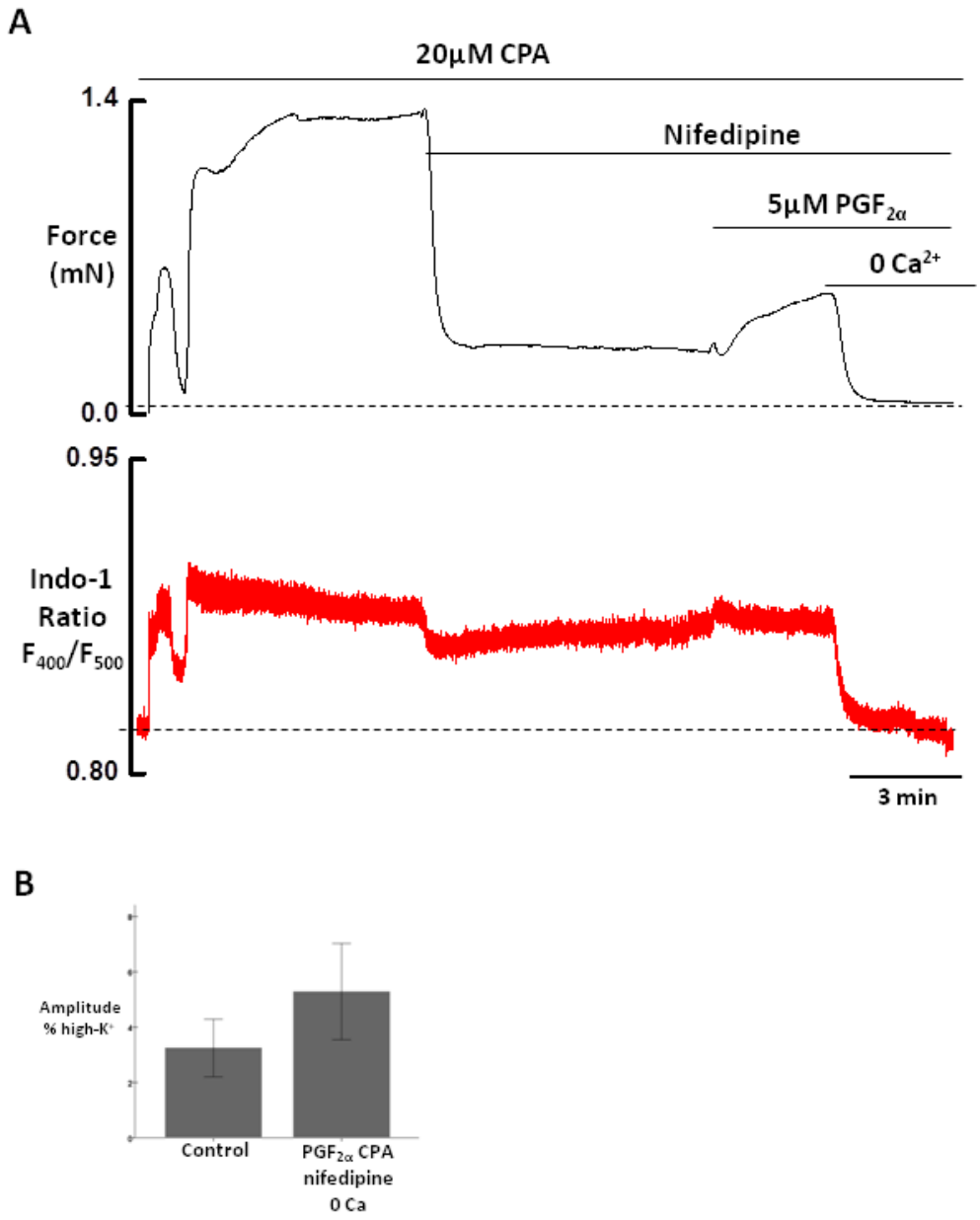

Figure 5.3.11.4 The importance of external Ca^{2+} in the effects of $PGF_{2\alpha}$ via ROC/NSCC. (A) Simultaneous recording of force (top trace) and $[Ca^{2+}]_i$ (bottom trace), showing that the force produced by ROC/NSCC is dependent on external Ca^{2+}. (B) Graph showing force generated by ROCC/NSCC in the absence Ca^{2+} ($n = 6$).

5.4 Discussion

Prostaglandin $PGF_{2\alpha}$ like other myometrial agonists, exert their effects through multiple mechanisms. They are able to increase $[Ca^{2+}]_i$ by release from the SR, which activates SOCE leading to the raised basal $[Ca^{2+}]_i$. It is also able to increase Ca^{2+} influx through a presently unknown ROC/NSCC pathway.

Preliminary evidence (Burdyga, T. unpublished) and previous work has shown that $PGF_{2\alpha}$ caused gradual depolarisation of the cell membrane leading to the initiation of trains of APs (Osa et al. 1983). The addition of $PGF_{2\alpha}$ resulted in a further increase in $[Ca^{2+}]_i$ and force when applied in high-K^+ depolarised tissue, in agreement with previous work (Ruttner et al. 2002). This increase in force and $[Ca^{2+}]_i$ could be caused by numerous mechanisms including activation or modulation of one or multiple plasma membrane ion channels, resulting in depolarisation of the membrane and enhanced Ca^{2+} influx through L-type VOCC. Previous work has shown the dependence of the effects of $PGF_{2\alpha}$ on external Ca^{2+} (Parkington et al. 1999; Coleman et al. 2000) however this is not the case, as $PGF_{2\alpha}$ is able to increase both $[Ca^{2+}]_i$ and force, when L-type VOCC are inhibited, as has been previously shown (Coleman et al. 2000). Whilst this cannot exclude the contribution of ion channel modulation of L-type VOCC, the degree of extra Ca^{2+} and force produced in the presence of nifedipine, and the lack of an increase $[Ca^{2+}]_i$ with $PGF_{2\alpha}$ highly suggests that other mechanisms are primarily responsible for the effects of $PGF_{2\alpha}$.

One of the mechanism by which agonists increase myometrial contractility is by release of Ca^{2+} from the SR, occurring through the classical GPCR pathway. Briefly GPCR upon binding to its agonist activates PLC, resulting in an increase in IP_3, releasing Ca^{2+} from the SR (Berridge et al. 1984). This occurs for oxytocin (Molnar et al. 1995) ATP (Gerwins et al. 1992)

and carbachol (Khac et al. 1996; Houdeau et al. 2005). $PGF_{2\alpha}$ is also able to release Ca^{2+} from the SR; in the absence of external Ca^{2+}, $PGF_{2\alpha}$ increased both $[Ca^{2+}]_i$ and force, which is in agreement with previous studies (Coleman et al. 1988; Coleman et al. 2000; Fu et al. 2000). $PGF_{2\alpha}$ presumably is able to do this using a mechanism similar to other agonists, including carbachol and ATP. Studies have shown that $PGF_{2\alpha}$ results in a dose dependent increase in IP_3. Unlike oxytocin, which is pertussis sensitive, $PGF_{2\alpha}$ is not and so is likely to be coupled to G_q, which is in agreement with FP data (Maka et al. 1993; Phaneuf et al. 1993; Coleman et al. 1994).

As $PGF_{2\alpha}$ releases Ca^{2+} from the store, it may be able to activate SOCE, allowing for a sustained influx of Ca^{2+} after depletion of the store. SOCE has been identified as an important mechanism of smooth muscle response to agonist in many tissue types; colonic (Kovac et al. 2008), cultured A10 cells (Xuan et al. 1992), coronary arteries (Wagner-Mann et al. 1992) and gallbladder myocytes (Morales et al. 2004), it has only recently been identified in myometrial smooth muscle, and studies are therefore limited (Wassdal et al. 1998; Shlykov et al. 2003; Noble et al. 2009; Murtazina et al. 2011).

Evidence of SOCE in smooth muscle comes from the SERCA inhibitors CPA and thapsigargin, both are essential tools in the investigation of SOCE, as it depletes the store without contamination from other downstream effects of agonists, such as ROC, NSCC and Ca^{2+} sensitisation. Both CPA and thapsigargin have been shown to deplete the SR and activate SOCE (Wayman et al. 1996a; Wayman et al. 1996b; Wayman et al. 1999) and have both been shown not to directly activate SOCE, with SR depletion critical to the activation of SOCE (Albert et al. 2002; Beech et al. 2004).

It has previously been reported in rat uterine smooth muscle that depletion of the store by CPA resulted in an influx of Ca^{2+} through SOCE, which is sensitive to the SOCE inhibitors SKF96365 and La^{3+}, while insensitive to nifedipine (Noble et al. 2009). The suggestion of SOCE in rat myometrial smooth muscle has been confirmed in this study, with the inhibition of L-type VOCC by nifedipine during CPA response there was a maintenance of both force and $[Ca^{2+}]_i$. There was also enhanced basal force and $[Ca^{2+}]_i$ after store depletion by CPA upon re-admittance of Ca^{2+} to the external solution. Both of these are highly suggestive of SOCE activity in uterine smooth muscle, due to the limited number of mechanisms that CPA can work through. The ability of the myometrium to operate through SOCE was further verified by the use of two non-specific SOCE inhibitors; La^{3+} and Gd^{3+} (Miyoshi et al. 2004; Noble et al. 2009) whereby enhanced rebound basal force was inhibited by both La^{3+} and Gd^{3+}, but not by the L-type VOCC inhibitor nifedipine.

While SOCE has been identified through $[Ca^{2+}]_i$ and force measurements in myometrial smooth muscle, the currents involved have not been studied to date, due in part to the difficulties in measuring store operated currents in excitable cells. The nature of the current involved in SOCE in myometrial cells remains undetermined, whether it is Ca^{2+} specific or involves multiple ions, as both Ca^{2+} specific currents (I_{CRAC}) and non-specific current (I_{SOC}) have been identified in response to store depletion in a number of tissue types (Vaca et al. 1994; Trepakova et al. 2000; Golovina et al. 2001; Albert et al. 2002). The identity of the channel or channels involved in SOCE in myometrial smooth muscle has also not been identified, but are likely to involve a heterogeneous group of ion channels. Studies have shown multiple proteins, mainly focusing on STIM, Orai and TRP family members, who are able to recreate both I_{CRAC} and I_{SOC} currents (Roos et al. 2005; Brueggemann et al. 2006;

Prakriya et al. 2006; Yeromin et al. 2006; Ambudkar et al. 2007). This heterogeneity in store operated channels may be responsible for the variation in the efficiency of SOCE inhibitors (Jung et al. 2003; Helli et al. 2005; McElroy et al. 2008) and their ability to block SOCE induced by different agonists (Murtazina et al. 2011).

The ability of myometrial smooth muscle to activate SOCE through agonists has been studied, albeit in a limited way, primarily with oxytocin (Wassdal et al. 1998; Murtazina et al. 2011) although other agonists such as bradykinin have been suggested to work through SOCE (Wassdal et al. 1998). Preliminary studies suggest both STIM, Orai and members of the TRP family are involved in the activation of SOCE in the myometrium (Shlykov et al. 2003; Murtazina et al. 2011). To date the ability of $PGF_{2\alpha}$ to induce SOCE has not been investigated, the ability to study this phenomenon induced by $PGF_{2\alpha}$ is made more difficult, due to its slow dissociation rate from its receptor (Coleman et al. 1994) and therefore long acting downstream effects, as shown in figure 4.3.4. This along with other mechanisms such as ROC, NSCC and Ca^{2+} sensitisation are responsible for the incomplete effects of La^{3+}. Inhibition of SOCE by La^{3+} while resulting in a substantial reduction in the enhanced basal $[Ca^{2+}]_i$ and force seen with $PGF_{2\alpha}$, did not completely abolish the response or stop the overlaying phasic contractility as seen with CPA, suggesting that other mechanisms are able to compensate partially if not fully for the effects of SOCE. These results suggest that while $PGF_{2\alpha}$ does induce SOCE, it is only one of a number of mechanism by which $PGF_{2\alpha}$ exerts it effects. In addition based on the lack of effects of Gd^{3+}, it does may not do this by the same channel as CPA induced SOCE.

As previously discussed it is difficult to separate SOCC from ROC and NSCC, but by utilising CPA and thapsigargin to empty the SR without activating receptors, the existence of such a

channel has been confirmed (Wayman et al. 1996a; Wayman et al. 1996b; Wallace et al. 1999; Wayman et al. 1999; Albert et al. 2002). There is also evidence for the existence of ROC, whether it is a Ca^{2+} selective or a non-specific channel, which are independent of store-operated channels. Firstly and the most convincing data is that depletion of the SR does not inhibit subsequent activation by agonists (Wang et al. 1991; Guibert et al. 1999; Zholos et al. 2004) and secondly inhibition of IP_3 receptors by heparin does not prevent ROC activation (Albert et al. 2003b).

While experimental evidence has inferred their existence, little is known about ROC in smooth muscle, especially that of the myometrium. It is not known if receptors are directly coupled to the ion channel, or if the actions are a result of downstream effects of PLC, as PLC inhibitors suppress many ROC (Beech et al. 2004) while DAG has been shown to directly activate NSCC in rat cardiomyocytes (Guinamard et al. 2004). In addition to the poor understanding of ROC, information regarding ROC is further compounded by NSCC. There is evidence that agonists are able to activate non-specific currents and that ROC are often non-specific in smooth muscle (Shimamura et al. 1994; Ma et al. 2008), it has not been determined if these are two separate entities or if there is cross over between the two. Due to this confusion, in this present study the contribution of ROC/NSCC to the effects of $PGF_{2\alpha}$ were not separated. When SOCE was maximally activated by CPA, $PGF_{2\alpha}$ resulted in an increase in $[Ca^{2+}]_i$ and force, although this statistical significance was not maintained when L-type VOCC was accounted for by nifedipine. As previously discussed this could have either been due to; a) the inherent problems of photometric measurements of $[Ca^{2+}]_i$ and the degree of variation within the experiment, or b) Ca^{2+} sensitisation. There is one previous study which suggests ROC as a mechanism of action of prostaglandin in guinea-pig

myometrium, although the individual contributions of SOCE, ROC and NSCC were not identified (Coleman et al. 2000). There is also evidence that external Na^+ is essential to the slow depolarisation phase of $PGF_{2\alpha}$ induced depolarisation, onto which the spike-like action potential bursts are superimposed (Reiner et al. 1976), Ca^{2+} sensitisation has also been suggested to be involved in the response of $PGF_{2\alpha}$ (Izumi et al. 1996; Woodcock et al. 2006). While this study suggests the importance of ROC/NSCC in the mechanism of $PGF_{2\alpha}$ it is impossible at this time to determine the contribution of ROC/NSCC and the current involved in the effects of $PGF_{2\alpha}$.

Whilst the evidence in this chapter is not conclusive, it strongly suggests that $PGF_{2\alpha}$ works through multiple mechanisms, as seen with other uterotonics. SOCE, ROC/NSCC all individually contribute to the effects of $PGF_{2\alpha}$, working in conjunction to increase myometrial mechanical output. Further study is needed to establish the contribution of these mechanisms in addition to Ca^{2+} sensitisation.

Chapter 6

Final discussion

Chapter 6

Final discussion

The aim of this work was to describe the relationship between force and $[Ca^{2+}]_i$ in rat late gestation longitudinal myometrial strips and the effects of $PGF_{2\alpha}$ on this relationship. The mechanism by which $PGF_{2\alpha}$ exerts its affects was also investigated, primarily focused on the role of Ca^{2+} entry mechanisms.

6.1 Force / Ca^{2+} relationship in pregnant rat uterine smooth muscle

Three physiological contractility profiles were determined for rat myometrial tissue. Two were spontaneously active, the first and most prevalent of the spontaneous tissue, gave smooth phasic contractions of; 1.31 mN, 15 second duration and a frequency of 5 contractions per minute. The second subset of spontaneously active tissue had irregular contractions, with contractions having a notched appearance and produced little force, with amplitude not reaching above 0.7 mN. The third and final contractility profile determined was quiescent; it was not spontaneously active but gave a normal contraction in response to high-K^+ depolarisation.

For all tissue, force mirrored Ca^{2+} transients, contractions were slightly preceded by a rise in $[Ca^{2+}]_i$, which remained raised for the duration of the contraction, falling before the relaxation phase. This rise in $[Ca^{2+}]_i$, was dependent on L-type VOCC, as inhibition with nifedipine resulted in abolishment of both spontaneous and high-K^+ induced activity, while gap junctions were needed for appropriate signal propagation.

This is in concurrence with the current understanding of the control of rat myometrial contractility and can explain the three contractility profiles found in pregnant rat uterine smooth muscle. Myometrial contraction is Ca^{2+}-dependent and is reliant upon the influx of Ca^{2+} through L-type VOCC, linked to the upstroke of action potentials (Shmigol et al. 1998b). When the slow wave of depolarisation reaches threshold potential for L-type VOCC, L-type VOCC open and the resulting influx of Ca^{2+}, is sufficient to activate calmodulin leading to the downstream phosphorylation of MLC_{20} and the production of force (Kuriyama et al. 1976). In rat uterine smooth muscle, action potentials are spike like (Mironneau 1973) and the characteristic of the train of action potentials are responsible for both Ca^{2+} and force characteristics (Burdyga et al. 2009).

The duration and frequency of myometrial contractions are modulated by action potentials. Modulation of membrane potential, alters the frequency that the threshold activation of L-type VOCC are reached, thus altering the frequency of Ca^{2+} transients and therefore frequency of contractions. While the duration of contractions are directly linked to the duration of the Ca^{2+} transient. Force is determined by the frequency of action potentials with the burst, with an increase in the frequency of action potentials giving greater force, as a result of summation (Mironneau 1973).

From this work in measuring simultaneous force and $[Ca^{2+}]_i$, it is clear that in understating the intimate relationship between membrane potential and Ca^{2+} parameters it is possible to infer information regarding membrane potential by monitoring changes in $[Ca^{2+}]_i$. Individual Ca^{2+} spikes within a Ca^{2+} transient are associated with single action potentials, with changes to the characteristics of bursts of action potentials, responsible for the change in Ca^{2+} characteristics and therefore force (Burdyga et al. 2009). This was shown particularly clearly

in irregular activity in figure 3.3.1.2 were the relationship between individual Ca^{2+} spikes to individual notches in force can be clearly seen. While more evidence would be gained from measuring simultaneous force, $[Ca^{2+}]_i$ and membrane potential, this is difficult and often impractical to do, but it is possible to infer information regarding membrane potential by monitoring changes in $[Ca^{2+}]_i$, although should be verified, by use of sucrose-gap simultaneous recording of electrical activity, Ca^{2+} and force.

6.2 The effect of $PGF_{2\alpha}$ on force /Ca^{2+} relationship in pregnant rat uterine smooth muscle

As with other uterotonics, $PGF_{2\alpha}$ resulted in an increase in the amount of force produced by the tissue, occurring in three characteristic ways. On spontaneously active tissue, $PGF_{2\alpha}$ had two responses; either increasing phasic activity, with an enhancement of both amplitude, duration and frequency or by inducing a sustained tonic-like contraction for the duration of agonist application. Meanwhile in quiescent tissue, $PGF_{2\alpha}$ initiated spontaneous contractility. In all tissue types once $PGF_{2\alpha}$ was withdraw, control parameters returned to the tissue after a lengthy washout period, suggesting a slow dissociation rate from the receptor, which has previously been described (Coleman et al. 1994).

As previously described, there is an intimate relationship between membrane potential, Ca^{2+} transients and the production of force in uterine smooth muscle. Similar to spontaneous contractility, $PGF_{2\alpha}$ induced contractions were preceded by a rise in $[Ca^{2+}]_i$, this Ca^{2+} transient remained for the duration of the contraction and was responsible for the frequency of contractions. Unlike frequency and duration, there was no change in the amplitude of $[Ca^{2+}]_i$, with $[Ca^{2+}]_i$ amplitude for spontaneous contractions the same as those

induced by $PGF_{2\alpha}$, and so was not responsible for the increase in mechanical output seen with the addition of $PGF_{2\alpha}$. The amplitude of contractions, as previously described is controlled by the frequency of action potentials and therefore Ca^{2+} spikes within the burst (Mironneau 1973; Kuriyama et al. 1976; Lammers et al. 1999; Burdyga et al. 2009). This could be clearly seen with the addition of $PGF_{2\alpha}$ onto spontaneously active tissue, were there was a significant increase in the frequency of Ca^{2+} spikes with the Ca^{2+} burst, correlating with the increase in force. In summary, from this work it can be concluded that it is the frequency of Ca^{2+} spikes with the Ca^{2+} burst is responsible for the amount of force produced in myometrial tissue, whether in spontaneous contractility or that induced by $PGF_{2\alpha}$. It is also probable that these Ca^{2+} spikes are directly controlled by the frequency of action potentials.

6.3 Mechanisms involved in the actions of $PGF_{2\alpha}$

While the importance of $PGF_{2\alpha}$ to myometrial contractility is in little doubt, little is known about its mechanism of action. Due to the importance of Ca^{2+} in the contractility of uterine smooth muscle, it was decided to investigate the three principal Ca^{2+} entry pathways activated in response to agonists in uterine smooth muscle; L-type VOCC, ROC/NSCC and SOCE.

6.3.1 The role of L-type VOCC in the effects of $PGF_{2\alpha}$

L-type VOCC play a pivotal role in both spontaneous and agonists induced contractility in pregnant rat myometrium, as they are responsible for the upstroke of the action potential.

A number of mechanism are able to modulate the Ca^{2+} influx through these channels in order to alter contractility (Brainard et al. 2007; Zhou et al. 2007). It is unlikely that $F_{2\alpha}$ is one of the agonists which do this, as firstly there was no change in the amplitude of $[Ca^{2+}]_i$ to reflect this, also inhibition of L-type VOCC by nifedipine did not prevent the increase in $[Ca^{2+}]_i$ and force produced by $PGF_{2\alpha}$. In addition when L-type VOCC were inhibited during $PGF_{2\alpha}$ induced contractions, while there was a significant reduction in both $[Ca^{2+}]_i$ and force, as would be expected due to the vital role of VOCC in any form of contraction in pregnant rat myometrium, a significant portion of both $[Ca^{2+}]_i$ and force remained. Based on this study it can be suggested that while $PGF_{2\alpha}$ does not modulate Ca^{2+} influx through L-type VOCC, while it is impossible to fully discount any contribution towards the effects of $PGF_{2\alpha}$, the contribution is likely to be negligible.

6.3.2 The role of ROC/NSCC in the effects of $PGF_{2\alpha}$

ROC is the term coined for an ion channel permeable to Ca^{2+} that is activated upon binding of a ligand to its receptor; whether that be directly coupled to an ion channel, such as the P2X receptor (Fredholm et al. 1994), or by downstream effectors, such as DAG (Guinamard et al. 2004). The currents invoked are often non-selective, in addition to Ca^{2+} they are often also permeable to both Na^+ and K^+, but are not voltage-sensitive, and are not activated by store depletion (Wray 1993). Difficulties arise trying to differentiate between ROC and NSCC as ROC can be non-specific, like NSCC, while NSCC can be activated in response to agonist stimulation, in addition to being found in control conditions (Kuriyama et al. 1995; Miyoshi et al. 2004). For ease of investigation in this preliminary study of the involvement of ROC

and NSCC in the mechanism of $PGF_{2\alpha}$, ROC and NSCC were not differentiated and treated as one entity.

CPA was used to determine if ROC/NSCC contribute to the effects of $PGF_{2\alpha}$. CPA inhibits the re-uptake of Ca^{2+} into the SR by SERCA, depleting the store, resulting in the activation of SOCE, as shown in this research and previously (Noble et al. 2009). In CPA contracting tissue, $PGF_{2\alpha}$ caused a further increased in both $[Ca^{2+}]_i$ and force above that produced by CPA alone. This increase in$[Ca^{2+}]_i$ and force could due to either ROC/NSCC. To discount the involvement of L-type VOCC, the inhibitor nifedipine was used. While the force remained significantly increased, $[Ca^{2+}]_i$ was no longer significantly higher than CPA alone, although this was likely due to a lack of sensitivity in the photometric system. Further work is needed to ensure that this lack of significance is not due to the inherent lack of sensitivity of measuring $[Ca^{2+}]_i$ by the photometric system. In order to determine the contribution of this pathway in the enhancement of force produced by $PGF_{2\alpha}$, electrophysiological techniques and further force Ca^{2+} experiments investigating the role of Na^+ and other ions need to be completed.

6.3.3 The role of Ca^{2+}-release Ca^{2+}-entry coupling in the effects of $PGF_{2\alpha}$

SOCE was identified over a quarter of century ago, suggesting that a specific plasma membrane channel is activated upon agonist stimulated depletion of the SR, resulting in an influx of Ca^{2+} (Putney 2011). It has now been identified in many cell types; including many smooth muscles, but investigations of SOCE in uterine smooth muscle are limited. In this study CPA and the SOCE inhibitors La^{3+} and Gd^{3+} were used to determine that SOCE does

occur in late gestation rat myometrial smooth muscle, as previously suggested (Shimamura et al. 1994; Fu et al. 2000; Noble et al. 2009).

Investigating SOCE induced by $PGF_{2\alpha}$ is complicated by the slow dissociation of the agonists from its receptor, contaminating results. From this work there are a number of lines of evidence to suggest that $PGF_{2\alpha}$ is able to induce SOCE. Critically like other myometrial agonists, $PGF_{2\alpha}$ releases Ca^{2+} from the SR, and so is a possible candidate for activating SOCE. In comparison with CPA, whose actions can only be due to SOCE, $PGF_{2\alpha}$ also resulted in a sustained rise in basal $[Ca^{2+}]_i$ and force when L-type VOCC were inhibited. Again similar to CPA there was a sustained enhanced rebound for both $[Ca^{2+}]_i$ and force with the re-admission of Ca^{2+} after store depletion, which was nifedipine insensitive, but attenuated with the SOCE inhibitor La^{3+}. These results infer the existence of SOCE induced by $PGF_{2\alpha}$, further work is needed to elucidate the mechanism behind SOCE in uterine smooth muscle, and what current and channels are involved.

6.4 Future work

The principal finding of this study was the identification of a significant release of Ca^{2+} from the SR, and a role for Ca^{2+} release Ca^{2+} entry coupling in the mechanism of $PGF_{2\alpha}$, primarily centred on SOCE, with the addition of Ca^{2+} influx through NSCC/ROC. It has left questions partially answered and opened up the possibilities of more mechanistic pathways in the involvement of $PGF_{2\alpha}$.

This study has focused upon the Ca^{2+} influx pathways and store release, in response to $PGF_{2\alpha}$. The release of Ca^{2+} from the SR in response of $PGF_{2\alpha}$ will not be without effects; Ca^{2+} can activate both K^+ channels and Cl^- channels, and agonists have been shown to modulate their function. Ca^{2+}-activated K channels; B_K, S_K and I_K can all be inhibited by TEA, and were shown not to be involved in the effects of $PGF_{2\alpha}$ (data not shown). The role of Cl_{Ca} channels has yet to be studied in response to $PGF_{2\alpha}$. It has previously been identified to increase contractility in response to oxytocin in uterine smooth muscle (Arnaudeau et al. 1994), and so may be an exciting mechanistic pathway for $PGF_{2\alpha}$ to exert its effects. In order to establish if Cl_{Ca} channels play a role in $PGF_{2\alpha}$ stimulation, simultaneous force and Ca^{2+} studies using the Cl_{Ca} channel inhibitor, niflumic acid, in addition to patch clamp studies need to be completed.

As previously discussed, Ca^{2+} sensitisation plays a role in agonist stimulation of uterine smooth muscle. Previous studies have suggested a role for Ca^{2+} sensitisation in the effects of $PGF_{2\alpha}$ in the uterus (Izumi et al. 1996; Woodcock et al. 2006), although further work needs to be completed in order to verify this and to determine the mechanism by which this occurs. In smooth muscle Ca^{2+} sensitisation occurs through modulation of either MLCP or MLCK, primarily through PKC or rho-kinase. Rho-kinase Ca^{2+} sensitisation occurring in

response to $PGF_{2\alpha}$ in rabbit aorta (Ito et al. 2003). In order to establish if this is involved in the mechanism of $PGF_{2\alpha}$, the rho-kinase inhibitor H1152 and the PKC inhibitor Ro-32-0432 and activator phorbol 12,13-dibutyrate (PDBu) in simultaneous force Ca^{2+} experiments would be completed.

SOCE and ROCE/NSCE have been identified to play a role in the increased mechanical output produced by $PGF_{2\alpha}$, the way in which both SOCE and NSCC/ROC function in smooth muscle are currently unknown. The current $PGF_{2\alpha}$ evokes through these pathways has not been identified in smooth muscle, presumably as a result of the difficulties in measuring small currents in excitable cells, were they can often be masked behind larger currents, and so difficult to isolate. Voltage-clamp studies on freshly isolated cells could be used to identify the currents induced by $PGF_{2\alpha}$, as it has previously been used to successfully identify a Na^{+} and Ca^{2+} dependent inward current induced by oxytocin in freshly isolated pregnant rat myocytes (Shimamura et al. 1994).

Identification of the channels involved in both SOCE and ROCE/NSCE, would be time consuming and difficult to achieve. A number of proteins have been proposed to form these channels. Perhaps the easiest to investigate would be STIM, which translocates to the PM upon activation (Zhang et al. 2005), and so immunohistochemistry techniques can be implemented in localise STIM in response to $PGF_{2\alpha}$. Orai and TRPCs have also been identified as possible SOCC and ROC/NSCC, use of RNAi introduced by reverse permeabilisation could be used to determine which proteins are involved in $PGF_{2\alpha}$ induced contractility, this technique has previously been used to knock down protein in intact vascular tissue, without affecting contractility (Corteling et al. 2007; Baeyens et al. 2010).

Another interesting possibility for a mechanism of $PGF_{2\alpha}$ is by increasing speed of propagation, by increasing interbundle communication. As shown in Chapter 3, gap junctions are pivotal to myometrial contractility, and have been shown previously to be modulated. There have been conflicting reports in regards to the modulation of gap junctions and prostaglandins (MacKenzie et al. 1983; Garfield et al. 1987), although no study has isolated $PGF_{2\alpha}$ from other prostaglandins, or investigated spread of propagation. In order to determine if $PGF_{2\alpha}$ influences gap junctions, confocal microscopy investigation of the temporal and spatial spread of Ca^{2+} through the tissue, along with simultaneous force recordings while inhibiting gap junctions the inhibitor 18-β-GA would need to be completed. In addition to this, to definitively exclude gap junctions from the cause of $PGF_{2\alpha}$'s effects, studies on isolated cells can be achieved using simultaneous electrophysiological, $[Ca^{2+}]_i$ and contractility in response to $PGF_{2\alpha}$ is possible.

6.5 Summary

The broad aim of this study was to determine the force / Ca^{2+} relationship induced by $PGF_{2\alpha}$. In order to determine this, firstly the force / Ca^{2+} relationship in pregnant rat myometrial tissue needed to be understood. Three contractility patterns were found; a) quiescent, not spontaneously active, but responsive to high-K^+ stimulation, b) spontaneous with irregular uncoordinated contractions, and c) spontaneous regular phasic contractions. Contractility was preceded by an increase in $[Ca^{2+}]_i$ and the initiation of Ca^{2+} spikes, which typically slowed before stopping, returning $[Ca^{2+}]_i$ to basal levels. This increase in $[Ca^{2+}]_i$ was dependent upon L-type VOCC, with synchronisation dependent upon gap junction propagation of action potentials.

$PGF_{2\alpha}$ increased mechanical output of all tissue types, by increasing the frequency of Ca^{2+} spikes within the Ca^{2+} burst. Quiescent tissue responded by the initiation of Ca^{2+} oscillations and concomitant force. While spontaneous tissue either resulted in an increase in phasic contractility or the production of a tonic-like contraction for the duration of agonist application.

The mechanism by which $PGF_{2\alpha}$ increases mechanical output is multifaceted. $PGF_{2\alpha}$ caused oscillatory release of Ca^{2+} from the SR in the form of propagating Ca^{2+} waves initiated at one end of the cell. The release from the store was sufficient to activate SOCE, shown as a nifedipine resistant Ca^{2+} influx which was sensitive to La^{3+}. In addition to the activation of SOCE, $PGF_{2\alpha}$ also activated a ROC/NSCC influx.

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